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THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1923

25042

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WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

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LEPTOSPIRAS, PATHOGENIC AND NON-PATHOGENIC.*

TOGETHER WITH SOME OBSERVATIONS ON THE SPIROCHETAL FLORA OF STAGNANT FRESH AND SALT WATER AND THE MAMMALIAM STOMACH.

BY HIDEYO NOGUCHI, M.D.

PLATES 1 to 5.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The genus *Leptospira*, of which *Leptospira icterohaemorrhagiae* may be taken as the type, is distinguished from other groups of minute spirochetes by its characteristic morphological and biological features. It is a delicate filament with tapered ends, so closely and regularly coiled as to resemble a rope. It is actively motile and shows a tendency to bend one or both ends, forming a C, S, 3, or J as it rotates in a fluid medium; in penetrating a semisolid medium its movements are serpentine, swiftly alternating forward, to the side, and backward, the body being flexible at any point and to any angle. No flagellum has been seen under the darkfield microscope, and preparations stained for flagella have so far given no satisfactory results. Preparations showing a single terminal flagellum at one or both ends appear to me unconvincing. In preparations stained by Miss Tilden the organisms gave the appearance of numerous peritrichal flagella of several microns in length along the entire length, and it is not improbable, in view of its characteristic movements, that the organism is provided with some flagella. Certain authors claim that there is a minute spherical body attached to one or both ends by means of an invisible thread, but I am unable to find any such structure. The leptospira resists the action of 10 per cent saponin. These characteristics are sufficient, I believe, to enable one to distinguish a leptospira from the multitude of minute forms of treponemata and other spirochetes.

* Read at the Annual Meeting of the Medical Society of the State of New York, at Albany, April 19, 1922.

Recognition of the existence of a new type of spirochete dates back to the time when a comparative study of the causative agent of infectious jaundice and of other spirochetes was undertaken by the writer (1918), and the creation of the new genus *Leptospira* for this rather important member of the family of pathogenic spirochetes seems to have been well justified, since at least two more pathogenic and several non-pathogenic varieties have now been added to this group. The pathogenic varieties at present known are *Leptospira icterohaemorrhagiae*, from infectious jaundice, *Leptospira hebdomadis*, from seven-day fever, a non-fatal disease present in Japan, and *Leptospira icteroides*, from yellow fever. Morphologically the three are difficult to differentiate, except that *Leptospira icteroides* is somewhat smaller than the other two. All three produce fever, hemorrhages, jaundice, and nephritis, individual symptoms differing in degree. Jaundice and nephritis are usually mild and are often absent in *Leptospira hebdomadis* infection; jaundice, nephritis, and fatty degeneration are more pronounced in the *icteroides* infection, while hemorrhage is predominant in the *icterohaemorrhagiae* infection. There are, however, border-line pictures of experimental infections such as are difficult to distinguish from one another, just as there are border-line cases of seven-day fever, infectious jaundice, and yellow fever. Serological differentiation of seven-day fever and infectious jaundice on the one hand and of infectious jaundice and yellow fever, on the other is possible by the Pfeiffer reaction, protection experiments, and to a certain extent by agglutination and complement fixation.

A very important contribution to our knowledge of infectious or epidemic types of jaundice has just been made by Wadsworth, Langworthy, Gilbert, Morris and Coleman.¹

The modes of transmission are somewhat different in the three forms of infection. The *hebdomadis* has been traced to field mice (*Microtus montebelli*) and the *icterohaemorrhagiae* to wild rats, both organisms being apparently harmless parasites in the kidneys of these animals and probably scattered by means of the urine. The infection of man is believed to occur through exposure of the skin to the contaminated

¹ Wadsworth, A., Langworthy, V., Stewart, C., Moore A., and Coleman, M. B.: Infectious Jaundice Occurring in New York State. *J. A. M. A.*, lxxviii, 1922, p. 1120.

water of sewers and cesspools. There are some authors, however, who do not exclude possible transmission by infected insects (mosquitos, horse flies, etc.), and experimental evidence has been adduced to show that these insects can transmit the infection, mechanically at least, soon after being fed on infected animals. In the case of yellow fever *Stegomyia fasciata* is the usual vector.

During the past few years a number of spirochetes resembling the leptospiras more or less in morphology have been described by European investigators. Hoffmann, in Germany, found in the dental deposits of normal individuals a small organism, which he designated *Leptospira dentium*, and which seems to be rather widespread among the inhabitants of Bonn (40 per cent). Uhlenhuth and Zuelzer demonstrated in the tap water of Berlin a spirochete morphologically indistinguishable from *Spirochaeta icterogenes* (a synonym for *L. icterohaemorrhagiae*) and gave it the name of *Spirochaeta pseudo-icterogenes*, since it was not pathogenic. Immunological differentiation of the *pseudo-icterogenes* from the *icterogenes* was not very striking. Hoffmann calls this organism *Leptospira aquatilis*. In this country, Wolbach and Binger described a similar organism, *Spirochaeta biflexa*, in the water of a stagnant pond near Boston.

Last summer it occurred to me that a study of water from various localities in a search for leptospiras might prove of interest. Samples of water from more or less stagnant ponds, swamps, and ditches were collected² in Woods Hole, Mass.; Mill Pond, Long Island; Newark Bay and Laurel Hill, N. J.; and from Shandaken, Hunter, and several other localities in the Catskill Mountains. Darkfield examination revealed the presence, usually in small numbers, of leptospira in all of the samples, (Figs. 1 to 9), although in some instances the number was so small that repeated examinations were necessary in order to find one organism. Both fresh and salt water contained organisms indistinguishable from *Leptospira icterohaemorrhagiae*; occasionally very short and minute (0.2×3 to 4 microns) (Fig. 1) and very heavy and long specimens (14 to 15 microns in length, maximum width 0.4 microns) were encountered. The spirals of the latter type were so

² The first leptospira-containing samples were those brought from Woods Hole by Miss Tilden and from Shandaken by the writer; the samples of fresh and salt water from the vicinity of New York City were collected by Mr. Klosterman and Mr. Farnan, also of the writer's laboratory.

tightly set together that the organisms appeared like a series of flat disks held in a row. These extremely small and large varieties are perhaps two different species, both differing from the *icterohaemorrhagiae* type. In addition to these varieties, leptospira-like forms without any perceptible elementary spirals, apparently smooth-bodied organisms (?) were observed; whether or not they were motile could not be determined. The movements of all the leptospiras found in water were rather sluggish. We have obtained growth of the water leptospiras in impure culture on our regular leptospira medium, though with considerable difficulty. Inoculations of the leptospira water samples into guinea pigs, white rats, and mice have been repeatedly made, but no infection could be induced in the animals. Injections of cultures likewise proved to be harmless. The kidneys and liver of the inoculated rats were removed after three weeks and suspensions of these organs injected into guinea pigs, with the hope that passage through rats might have enhanced the virulence of the organisms, but so far no positive results have been obtained; the water leptospiras appear to be non-pathogenic for guinea pigs as well as rats. Since we have no pure cultures no attempts have yet been made to establish the immunological relationships of the pathogenic and water varieties of leptospira.

In passing, it may be of interest to note the varieties of spirochetes which we encountered in studying the water samples. We found, besides those already described, organisms belonging to the *plicatilis*, (Figs. 10 to 14, and 18 to 25), the *buccalis* and *refringens*, (Figs. 26 and 27) the *macrodentium*, (Figs. 16 and 17) and the *microdentium* (Fig. 31) types, as well as several spirilla, (Figs. 40 to 43) including the widespread *Spirillum undulans*. All were obtained by Miss Tilden in impure culture on the leptospira medium (at room temperature).

Although unsuccessful in finding a leptospira in the human mouth, I detected a minute leptospira in the gastric mucosa of the ox (*Leptospira bovis*, n. sp., Fig. 50). Examination of the contents and mucous membranes of the stomach of dogs, cats, sheep, pigs, rabbits, rats and guinea pigs did not reveal any leptospira. In the stomach of the sheep, (Fig. 49) ox and cat (Figs. 46 to 48), Dr. Howard B. Cross, who also assisted in this work, found a number of spirochetes belonging to the *microdentium* type, some of which simulated the leptospira, though distinguishable without difficulty.

EXPLANATION OF PLATES.

PLATE 1.

Magnification $\times 1,000$.

Non-pathogenic leptospiras from fresh and salt water.

FIGS. 1 to 5. Darkfield views of the very small type of leptospira, Fig. 1, and of the average type, Figs. 2 to 5, which corresponds in size with *Leptospira icterohaemorrhagiae*.

FIG. 6. Darkfield view of a very much coarser and somewhat more loosely coiled organism which may or may not belong to the leptospira group.

FIGS. 7 to 9. Water leptospiras stained by Miss Tilden's modification of Loeffler's method. Figs. 7 and 8 are of average size, Fig. 9 is somewhat heavier.

PLATE 2.

Magnification $\times 1,000$.

Water spirochetes of various types.

FIGS. 10 to 14. Large spiral organisms of the type of *Spirochaeta plicatilis*, as seen by darkfield and in preparations stained by Miss Tilden's modification of Loeffler's technique for flagella staining. Compare with Migula's photographs of *Spirochaeta plicatilis*, Figs. 34 and 35.

FIG. 15. Compare with Zuelzer's photographs of *Spirochaeta plicatilis*, Figs. 37 to 39; the latter are however magnified 1,500 times (instead of 1,000).

FIG. 16. Darkfield view of a treponema of the *macrodentium* type.

FIG. 17. Probably the same type. Stained by Miss Tilden's modification of Loeffler's technique for flagella staining.

FIGS. 18 to 25. Spiral organisms of the smaller *plicatilis* type. Compare with Migula's *Spirochaeta plicatilis*, Fig. 34, and also with Ehrenberg's drawing, Fig. 33. The stained preparations were made by Loeffler's technique for flagella staining.

FIGS. 26 and 27. Indeterminate types, somewhat resembling *Treponema buccale* or *Treponema refringens*.

FIGS. 28 to 30. Indeterminate types, stained by Miss Tildens' modification of Loeffler's technique for flagella staining.

FIG. 31. A treponema of the *microdentium* type.

FIG. 32. A treponema apparently intermediate between the *microdentium* and *macrodentium* types.

PLATE 3.

Photographs and drawings of *Spirochaeta plicatilis* for comparison with Figs. 10 to 32. ♦

FIG. 33. Ehrenberg's drawings of *Spirochaeta plicatilis*. The upper figures were said by him to represent a magnification of 300 times, the lower a magnification of 800 times.

FIG. 34. *Spirochaeta plicatilis* according to Migula, stained with gentian violet. Magnification $\times 1,000$.

FIG. 35. *Spirochaeta plicatilis* according to Migula, stained by Loeffler's technique for flagella staining. Magnification $\times 1,000$.

FIG. 36. Schaudinn's schematic drawing of a portion of *Spirochaeta plicatilis*.

FIGS. 37 to 39. *Spirochaeta plicatilis* according to Zuelzer, stained with iron hematoxylin, and showing the axial filament. Magnification $\times 1,500$.

PLATE 4.

Magnification $\times 1,000$.

FIGS. 40 to 43. Darkfield views of a culture of a spirillum from fresh water.

PLATE 5.

Magnification $\times 1,000$.

Spirochetes found in the stomach of normal animals.

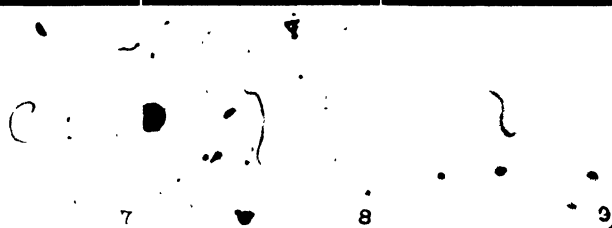
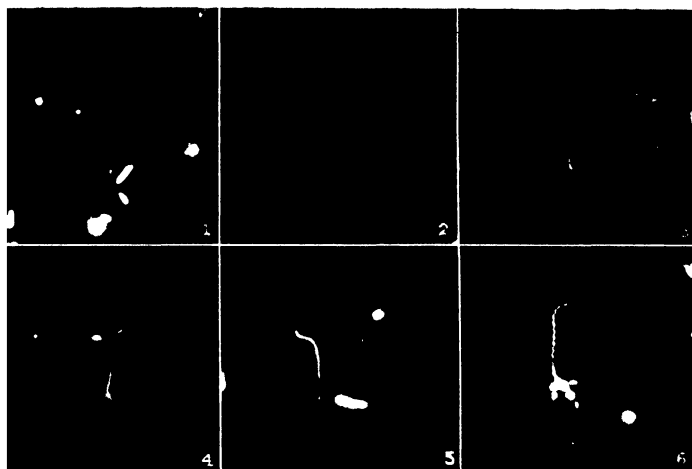
FIGS. 44 and 45. Darkfield views of spirilla found in the stomach of the dog.

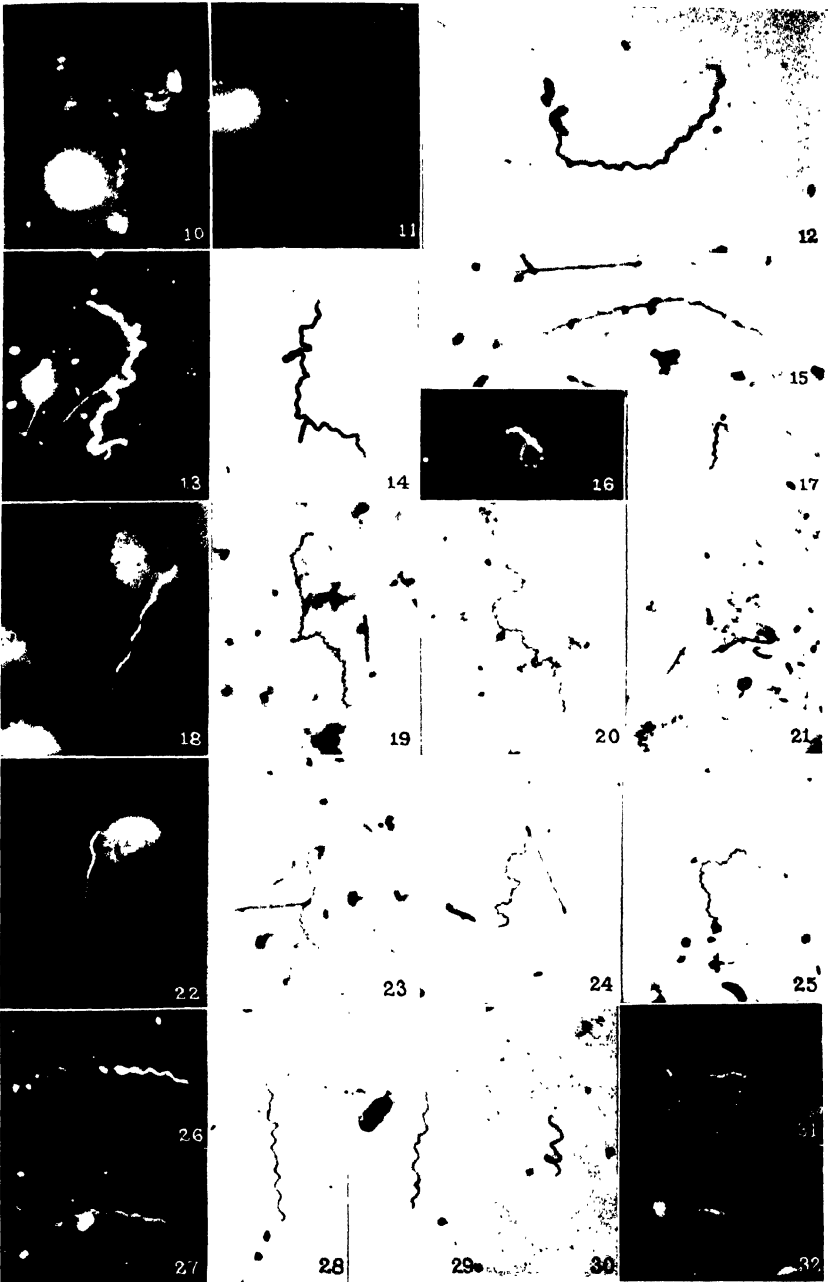
FIG. 46. A similar spiral organism from the cat's stomach. Darkfield view.

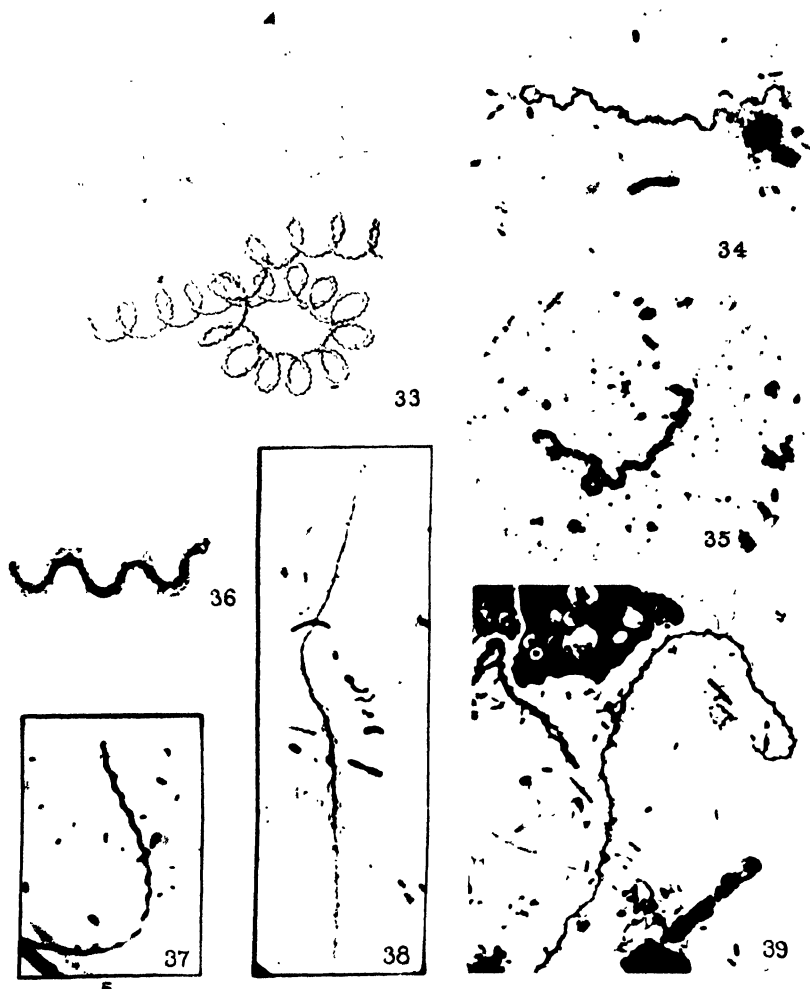
FIGS. 47 and 48. Darkfield views of treponemata of the *microdentium* type found in the cat's stomach.

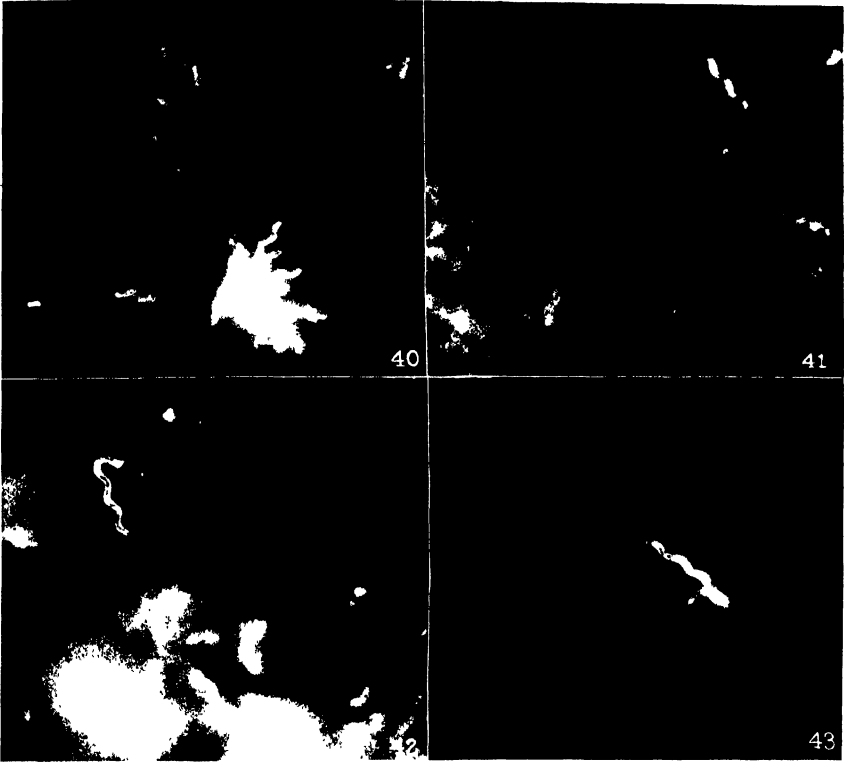
FIG. 49. Darkfield view of a treponema of the *microdentium* found to be very common in the stomach of the sheep.

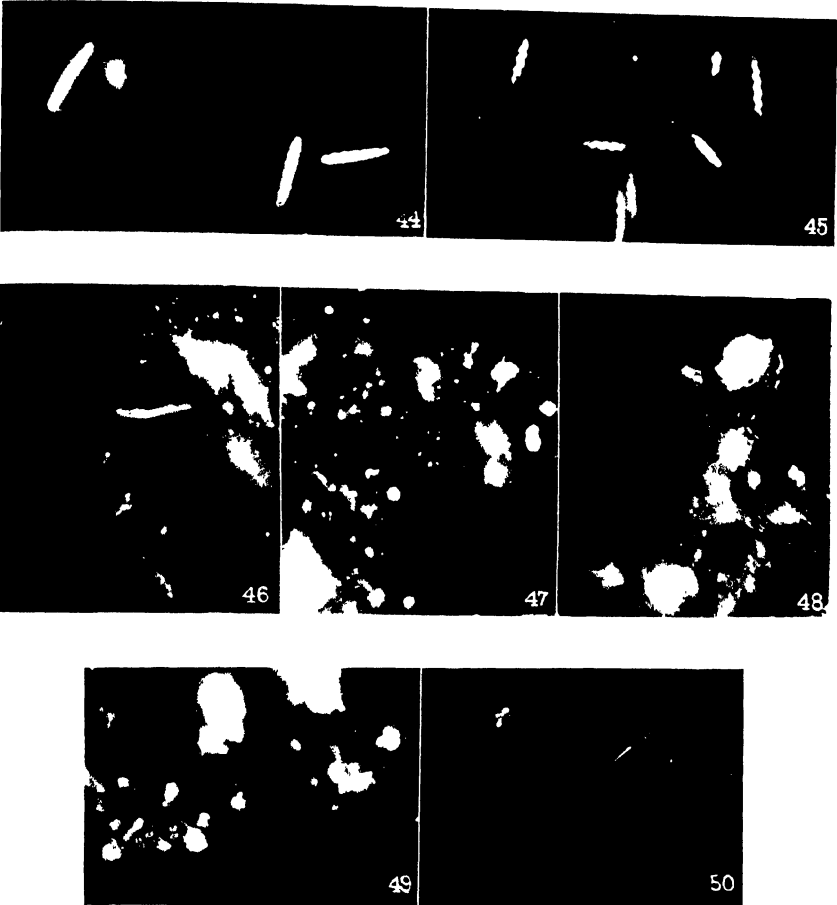
FIG. 50. Darkfield view of the minute leptospira (*Leptospira bovis*, n. sp.) found in small numbers in the stomach of the ox.











BLOOD DESTRUCTION DURING EXERCISE.

I. BLOOD CHANGES OCCURRING IN THE COURSE OF A SINGLE DAY OF EXERCISE.

BY G. O. BROUN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 13, 1922.)

The studies of Rous and Robertson¹ have shown that normally the destruction of erythrocytes is accomplished, in part at least, by a process of fragmentation in the blood stream. There is evidence that the breaking up is the result of mechanical injury. Among physiologic conditions which would favor such disintegration, vigorous exercise at once suggests itself. Both the increased rapidity of blood flow and the violent muscular contractions would tend to increase the wear and tear upon the corpuscles. The present investigations have been undertaken with the object of determining whether an increased rate of blood destruction during exercise could be demonstrated.

Since the subject is closely linked with the general question of fluid and cell changes during exercise, a number of observations have been made in this connection, and it is with these that the present paper will be largely concerned. The data given serve as controls to the method employed in experiments subsequently to be reported.

The changes in the blood during exercise are not yet completely understood. The early literature upon the subject has been well summarized by Hawk.² Practically without exception, investigators have reported an increase in hemoglobin and corpuscles immediately after short periods of exercise. Schneider and Havens³ confirmed these findings in observations made at low altitudes, but following exercise at high altitudes found no increase in either cells or pigment. On the

¹ Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

² Hawk, P. B., *Am. J. Physiol.*, 1903-04, x, 384.

³ Schneider, E. C., and Havens, L. C., *Am. J. Physiol.*, 1914-15, xxxvi, 380.

other hand, Cohnheim and his coworkers⁴ as well as Gross and Kestner⁵ have shown an actual decrease in red count and per cent of hemoglobin after long and strenuous mountain climbing. This they attribute to dilution of the blood with fluid derived from the muscles. The workers who have found a concentration of the blood during exercise explain it either by a changed distribution of the corpuscles in the vessels² or by loss of fluid into the muscles⁶ or liver lymphatics.⁷

Obviously the points at issue can only be cleared up by accurate determinations of cell and plasma volume during exercise. So far as we are aware, no such observations have been reported. The lack of a method of estimating blood volume which is at once accurate and innocuous has no doubt hitherto prevented advancement along this line.

Method.

Two types of blood volume determination have shown themselves to be of practical value for use on the living animal. The first is the well known carbon monoxide inhalation method, originally devised by Gréhant and Quinquaud.⁸ The second entails the addition of some substance to the blood plasma and the subsequent determination of its degree of dilution. Perhaps the most satisfactory of the numerous substances employed in this way is the dye, vital red, first used by Keith, Rowntree, and Geraghty.⁹ The non-toxic nature of the dye allows repetition of the volume determinations at short intervals. Since the carbon monoxide method cannot readily be repeated within a single day, the dye method was adopted for use in the present series of experiments.

The technique followed closely that described in detail by Hooper, Smith, Belt, and Whipple¹⁰ and Smith.¹¹ Mention need be made only of slight modifications.

⁴ Cohnheim, O., Kreglinger, and Kreglinger, *Z. physiol. Chem.*, 1909, lxxiii, 413.

⁵ Gross, W., and Kestner, O., *Z. Biol.*, 1920, lxx, 187.

⁶ von Willebrand, E. A., *Skandin. Arch. Physiol.*, 1903, xiv, 176.

⁷ Lamson, P. D., *J. Pharmacol. and Exp. Therap.*, 1920-21, xvi, 125.

⁸ Gréhant and Quinquaud, E., *Compt. rend. Acad.*, 1882, xciv, 1450.

⁹ Keith, N. M., Rowntree, L. G., and Geraghty, J. T., *Arch. Int. Med.*, 1915, xvi, 547.

¹⁰ Hooper, C. W., Smith, H. P., Belt, A. E., and Whipple, G. H., *Am. J. Physiol.*, 1920, li, 205.

¹¹ Smith, H. P., *Am. J. Physiol.*, 1920, li, 221.

Vital red was used in 1 per cent strength for injection, and dogs of 10 to 15 kilo body weight received 4 cc. of it, while those above 15 kilos received 5 cc. In making the dilute dye solution for the preparation of a standard, 1 cc. of the 1 per cent dye solution was made up with distilled water to 250 cc. in a volumetric flask. This gave a solution of the strength of $1/25,000$, a figure easier to deal with than the fraction $1/26,666.67$ employed in the articles referred to above. It was found to be quite important to allow the blood to resume its normal flow for several minutes after the manipulations necessary to the introduction into the vein of the aspirating needle, since stasis caused by pressure on the vessel changes the hematocrit value. The procedure employed was as follows:

The needle, firmly grasped at the base by a long jawed hemostat, is connected to a syringe containing a little saline solution. While an assistant makes pressure upon the external jugular vein just above the clavicle, the needle is thrust into its lumen. The aspiration of blood into the saline solution shows clearly when the needle is in place. Pressure on the vein is then discontinued, the syringe is detached, leaving the needle in place, and a close fitting stylet is introduced into the lumen of the latter, completely closing it. After 2 or 3 minutes have been permitted to elapse, during which presumably the normal flow in the vein is completely reestablished, the stylet is withdrawn and a sample of blood taken into a syringe. The same method is used in obtaining both specimens required for each volume determination. In the case of the second specimen it has the special advantage of insuring the accuracy of the 4 minute period between the dye injection and the withdrawal of the blood.

The hematocrit determinations were made by filling three tubes of the type described by Epstein¹² from each specimen of blood and centrifuging these at 3,500 revolutions per minute for 30 minutes. The average plasma per cent of the three tubes, after correction for oxalate dilution, was taken as the true plasma per cent of the blood. To obtain the plasma for colorimetric readings, the centrifuge tubes were centrifuged at 2,000 revolutions per minute for 10 to 15 minutes. This modification, while adding somewhat to the complexity of the method, has given more uniform results than the use of graduated centrifuge tubes.

Hemoglobin determinations were made by the method of Newcomer.¹³ The colored glass standard had been checked against gasometrically standardized Palmer¹⁴ and Robscheit¹⁵ solutions and found to correspond closely to these throughout a wide range. The specimen for hemoglobin was withdrawn from the oxalated blood of the jugular vein as obtained for the volume determinations. The reading was subsequently corrected for dilution of blood with oxalate. Per cent of hemoglobin is given in terms of the Haldane scale. 100 per cent hemoglobin on this

¹² Epstein, A. A., *J. Lab. and Clin. Med.*, 1915-16, i, 610.

¹³ Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.

¹⁴ Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

¹⁵ Robscheit, F. S., *J. Biol. Chem.*, 1920, xli, 209.

scale is equivalent to an oxygen-carrying capacity of 18.5 cc. per 100 cc. of whole blood. This corresponds to 13.8 gm. of hemoglobin per 100 cc. of whole blood.

The few red counts recorded were made in duplicate from the oxalated blood of the jugular vein, subsequently corrected for oxalate dilution. Hayem's fluid was used and the Bürker-Zeiss counting chamber.

In reporting the results the term "pigment volume" has been used in the sense in which Whipple and his coworkers¹⁶ employed it; namely, as the product of the blood volume by the per cent of hemoglobin. Subject to the obvious limitations of the vital red method, it will serve as an index of the total circulating blood pigment. But since the hematocrit and hemoglobin values of blood specimens withdrawn from the jugular vein are influenced by changes in cell distribution, this factor must be borne in mind in interpreting the results.

Experimental Procedure.

The chief reason for choosing dogs as the experimental animals, apart from their general utility, was the special susceptibility of their red corpuscles to mechanical injury.¹⁷ It seemed probable that because of this characteristic the blood destruction during exercise would be greater than in the other available species.

The animals were kept in individual cages and fed upon a mixed diet containing considerable meat. To avoid possible changes in blood volume resulting from the ingestion of food, food was not given upon the day of the experiment until after the last blood volume had been completed. But it was found necessary to allow water freely.

For purposes of exercise, treadmills were used, so constructed that the tread was at an angle of 20° to the horizontal. A bicycle cyclometer attached to the machine recorded the number of miles traveled. The standard day's exercise consisted of two periods, each of 2 hours, separated by a rest of 1 hour. The distances covered during these periods varied considerably, being chiefly limited by the ability of the animals to withstand the exertion. All of the animals worked actively, yet the average per day for the series was only 3.8 miles. This may

¹⁶ Whipple, G. H., Hooper, C. W., and Robscheit, F. S., *Am. J. Physiol.*, 1920, liii, 151.

¹⁷ Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

seem surprisingly low, but the fact should be recognized that the 20° inclination of the tread necessitated a vertical ascent of 6,870 feet in this distance. A careful oversight was maintained to prevent exhaustion. Except in the initial period of 10 to 15 minutes exercise, no attempt was made to insure a continuous run, brief rest being allowed as needed. However, immediately before the last of the three blood examinations, the animal was run continuously for about 10 minutes.

It should be noted that the twelve complete experiments here reported were all done during warm weather and panting was regularly induced.

Two series of experiments were performed which will be designated respectively the "exercise series" and the "control series."

Three animals, Nos. 3, 4, and 7, appear in both series of experiments, and in the exercise series Nos. 3 and 5 appear twice. The repetition of experiments on the same animal was in most cases carried out only after the lapse of a considerable time, and during the interval some change in body weight occasionally occurred. The experiments on No. 8 and the first of the two on No. 5 were made when the animals had not completely recovered from an anemia induced by an hemolytic serum. The conditions in these cases were not entirely normal, yet the blood changes noted are of the same general character as those of the other animals of the series. We have, however, deemed it advisable to list the results with them separately in Table I, which shows the "normal" blood findings of the animals. All of the other animals were in good health at the time of observation.

The exercise experiments were conducted in the following manner. The dog was removed directly from cage to table and the first blood volume specimens were taken. From 15 to 20 minutes later the animal was placed on the treadmill and exercised vigorously for 10 to 15 minutes, then quickly removed, and a second set of determinations on the blood made at once. Following this the animal was exercised for 2 hours, allowed to rest for 1 hour, and again exercised for another 2 hour period. Finally, at the end of this time, the third blood volume determination was made. This procedure was slightly altered in the case of Dogs 1 and 7 which were exercised for 3 consecutive hours between the second and third blood volume determinations, without rest or access to water. In the first experiment on Animals 3 and 5, and in the single experiment on Animals 6 and 8, the blood was examined before exercise and after 10 minutes exercise. A third examination was not made.

In the control series the three blood examinations were made at approximately the same intervals as in the exercise series. In the case of Dogs 10 and 3, only two determinations were carried out, an interval of 30 minutes separating those of Dog 10 and 6 hours those of Dog 3.

We have purposely omitted the following blood volume determinations of Animals 5 and 8 from the normal series as the animals had not entirely recovered from anemia due to the injection of an hemolytic serum. Their reactions to exercise did not differ from those of normal animals.

	5	14	941	6.72	328	2.34	1,269	9.06	2 mos. previous to observation noted above.
	8	10½	849	8.29	405	3.95	1,254	12.24	

The Blood in the Resting Animal.

The findings in the initial blood specimen in both series of animals are given in Table I. This specimen is assumed to yield the normal relation of the blood components during rest. The figures obtained compare closely with those reported for normal dogs by Smith, Arnold, and Whipple.¹⁸ The average cell volume is somewhat smaller and the plasma volume slightly larger than they found by the use of the dye method. The cell volume figures, however, correspond almost exactly to their determinations made by the carbon monoxide method, which they regard as yielding figures that closely approximate the true cell volume. The accompanying high hemoglobin content of the blood shows that the differences noted are not due to anemia.

The Effect of Brief Exercise.

The changes which occurred during exercise are recorded in Table II and are graphically shown in Text-fig. 1. Since the first examination of the blood is taken to indicate the normal condition in each animal while resting, there is shown in the table to the right of the actual figure for each determination its percentage in terms of the findings at the first determination. In Text-fig. 1 the percentage changes in each of the blood components are shown and compared with the initial finding.

It will be noted that 10 to 15 minutes of active exercise caused usually a slight increase in plasma volume and quite regularly a marked increase in cell volume, hemoglobin, pigment volume, and number of corpuscles per cubic millimeter. For the series there is an average increase in plasma of 3.6 per cent. In two individuals it showed a slight decrease. The cell volume increased in every case, averaging 12.3 per cent, with a variation from 1.5 per cent to 24.0 per cent. Pigment volume follows closely the changes in cell volume, the increase ranging from 5 to 25 per cent with an average of 16.1 per cent. The per cent of hemoglobin shows a maximal rise of 13 and a minimal of 2, the average being 8.5. The increase in number of red corpuscles per cubic millimeter averages 11 per cent for the two instances followed.

¹⁸ Smith, H. P., Arnold, H. R., and Whipple, G. H., *Am. J. Physiol.*, 1921, lvi, 336.

The Changes Consequent on More Prolonged Exercise.

After several hours of exercise, the plasma volume shows consistently an increase. The average rise above the resting normal is 11.7 per cent, with minimal and maximal variations of 6.8 and 18 per cent. The average increase over the plasma volume after 10 minutes of exercise is 8.1 per cent. In one instance there is a decrease in plasma volume, but of less than 1 per cent, between the second and third determinations. The greatest increase observed is 13 per cent. On the other hand, cell volume, while remaining at an average of 9.4 above the resting cell volume, shows an average decrease of 2.9 per cent below the average found at the end of 10 minutes exercise. In two instances there are increases of 1 and 2.5 per cent. The tendency of the hemoglobin percentage to decrease is more marked but this is, in part at least, due to dilution of the blood by increase in plasma volume. However, the calculated pigment volume, in which this error of dilution is corrected, shows after the period of prolonged exercise a 7.7 per cent fall below the average after 10 minutes of exercise. In no instance is an increase to be found. The final average pigment volume is, however, 8.4 per cent above the average of the first determination. The red count in the two instances followed shows a marked decrease after the prolonged exercise, part of which may be ascribed to the dilution of the blood referred to above.

The Control Series.

Although the animals used as controls were kept in small cages throughout the periods of observation, it was not possible to prevent a certain amount of activity on their part. The dogs varied much in this respect. It is not surprising, then, that the results obtained vary somewhat too (see Table III and Text-fig. 2). Nevertheless they are in striking contrast with those of the exercise series (compare Tables II and III). There is an average increase of 4.6 per cent in the second plasma volume over the first, an increase which closely corresponds to that observed in animals exercised for 10 minutes. It is quite possible that in both series a portion of this increase may be due to the disturbing influence of the first blood volume determination, in which approximately 20 cc. of blood is withdrawn and 4 to 5 cc.

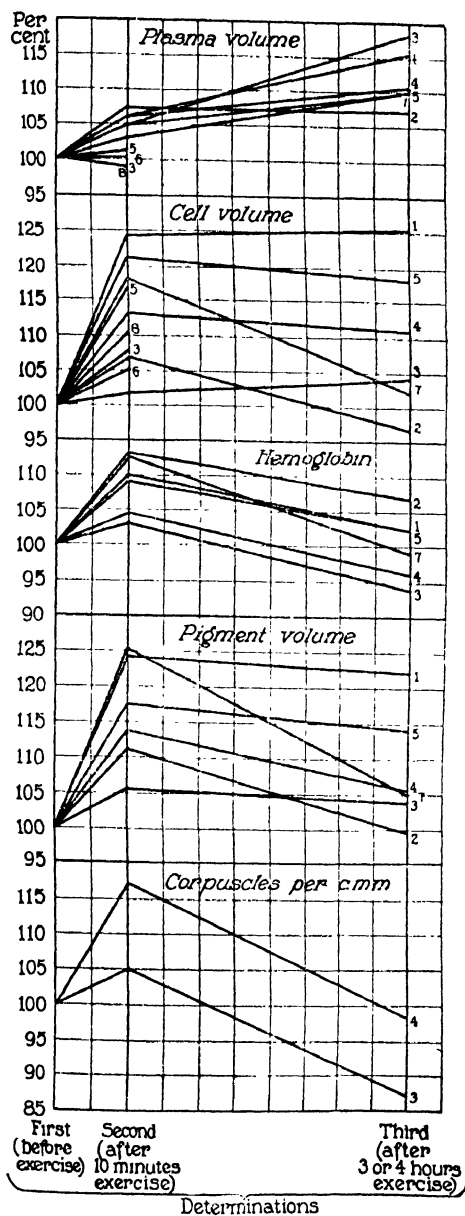
TABLE II.
The Changes during Exercise.

Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			Cc.	In percentage of the first given.	Cc.	In percentage of the first cell volume here given.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here given.	Per c. mm.	In percentage of the first red count here given.
1. Female collie; weight 20½ kilos.	0	Before exercise.	1,078	100.0	782	100.0	111.0	100.0	2,062	100.0		
	30 min.	After 10 min. exercise.	1,140	106.0	970	124.0	122.0	110.0	2,570	124.0		
	3 hrs., 30 min.	After 3 hrs. exercise. Had run 3 miles. No rest period.	1,242	115.0	979	125.0	113.0	102.0	2,512	122.0		
2. Male mongrel; weight 14½ kilos.	0	Before exercise.	923	100.0	717	100.0	119.0	100.0	1,950	100.0		
	30 min.	After 10 min. exercise.	992	107.5	764	106.5	123.0	113.0	2,160	111.0		
	3 hrs., 30 min.	After 4 hrs. exercise. Had run 4 miles in all with an interpolated hr. of rest.	985	106.8	691	96.5	116.0	106.5	1,944	99.8		

3. Male foxhound; weight 20½ kilos.	0 30 min.	Before exercise.	1,244	100.0	936	100.0							
		After 10 min. exercise.	1,234	99.5	1,006	107.5							
		cise.											
3 mos. later; weight 18½ kilos.	0 30 min.	Before exercise.	1,190	100.0	968	100.0	118.0	100.0	2,548	100.08,600,000	100.0		
		After 10 min. exercise.	1,249	105.0	981	101.5	120.5	103.2	2,690	105.59,000,000	105.0		
		cise.											
4. Male pointer; weight 18½ kilos.	5 hrs., 30 min.	After 4 hrs. exercise. Had run 5 miles in all with an interpolated hr. of rest.	1,405	118.0	1,005	104.0	110.0	93.5	2,650	104.07,500,000	87.3		
5. Male collie; weight 14 kilos.	0 30 min.	Before exercise.	1,226	100.0	818	100.0	113.0	100.0	2,310	100.06,100,000	100.0		
		After 10 min. exercise.	1,298	106.0	922	113.0	118.0	104.5	2,620	113.57,130,000	117.0		
		cise.											
3 mos. later; weight unchanged.	5 hrs., 30 min.	After 4 hrs. exercise. Had run 5 miles in all with an interpolated hr. of rest.	1,352	110.5	909	111.0	108.0	95.7	2,440	105.56,000,000	98.5		
5. Male collie; weight 14 kilos.	0 30 min.	Before exercise.	941	100.0	328	100.0							
		After 10 min. exercise.	955	101.5	382	116.5							
		cise.											
3 mos. later; weight unchanged.	5 hrs., 30 min.	Before exercise.	940	100.0	408	100.0	89.0	100.0	1,200	100.0			
		After 10 min. exercise. Had a 1 hr. rest period at noon.	965	103.0	493	121.0	96.8	109.0	1,410	117.5			
		After 4 hrs. exercise. Had run 3 miles.	1,033	110.0	483	118.0	90.5	102.0	1,372	114.0			

TABLE II—*Concluded.*

Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			Cc.	In percentage of the first given.	Cc.	In percentage of the first cell volume here given.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here given.	Per c. mm.	In percentage of the first red count here given.
6. Male setter; weight 20½ kilos.	0 30 min.	Before exercise. After 10 min. exercise.	983	100.0	967	100.0						
			990	100.5	1,025	105.0						
7. Male mongrel; weight 18½ kilos.	0 30 min.	Before exercise. After 10 min. exercise. No rest period.	1,030	100.0	812	100.0	112.0	100.0	2,062	100.0		
			1,080	105.0	960	118.0	126.0	112.5	2,570	125.0		
8. Male bull mongrel; weight 10½ kilos.	3 hrs., 30 min.	After 3 hrs. exercise. Had run 3 miles.	1,132	110.0	830	102.0	110.0	98.5	2,160	105.0		
	0 4 hrs.	Before exercise. After 10 min. exercise.	849	100.0	405	100.0						
			845	99.5	447	110.5						

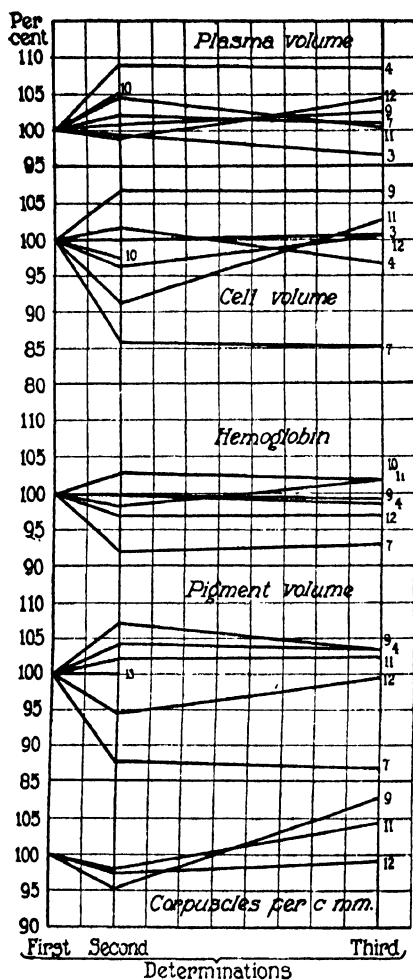


TEXT-FIG. 1. Percentage changes in the blood components during exercise.

TABLE III.
Control Findings.

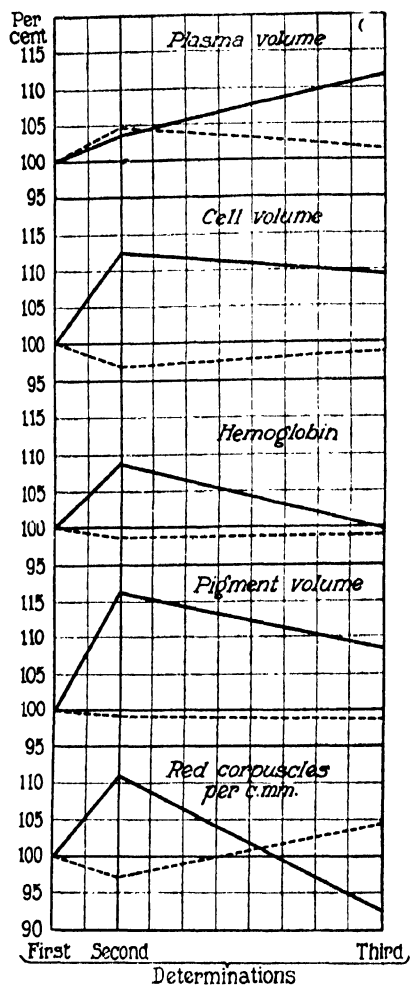
Animal.	Elapsed time.	Circumstances.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles.	
			C.	In percentage of the first given.	C.	In percentage of the first.	Per cent.	In percentage of the first hemoglobin determination here given.	Units.	In percentage of the first pigment volume here given.	Per c. mm.	In percentage of the first red count here given.
9. Male mongrel; weight 11½ kilos.	0	No exercise on this day.	703	100.0	507	100.0	118.0	100.0	1,437	100.0	8,000,000	100.0
	30 min.		708	100.8	544	107.0	119.0	100.9	1,490	104.0	7,600,000	95.0
	5 hrs., 30 min.		717	102.0	543	107.0	117.5	99.6	1,480	103.0	8,600,000	107.2
10. Male mongrel; weight 14½ kilos.	0	No exercise on this day. Only two blood volume determinations made.	719	100.0	723	100.0	127.0	100.0	1,832	100.0		
	30 min.		752	105.0	716	97.7	125.0	98.5	1,832	100.0		
	5 hrs., 30 min.						130.0	102.0				
11. Male mongrel; weight 14½ kilos.	0	No exercise on this day.	736	100.0	563	100.0	111.0	100.0	1,440	100.0	7,800,000	100.0
	30 min.		770	104.5	514	91.5	114.0	103.0	1,465	102.0	7,600,000	97.5
	5 hrs., 30 min.		730	99.8	581	103.0	112.0	102.0	1,490	102.0	8,100,000	103.8

	0	No exercise on this day.	692	100.0	610	100.0	134.0	100.0	1,748	100.0	8,200,000	100.0
12. Male mongrel; weight 11½ kilos.	30 min.											
	5 hrs., 30 min.											
3. Male foxhound; weight 20½ kilos.	0	No exercise on this day.	1,174	100.0	894	100.0						
	6 hrs.	Only two determinations made.	1,127	96.0	911	101.0						
4. Male pointer; weight 20½ kilos.	0	No exercise on this day.	1,402	100.0	683	100.0	88.0	100.0	1,835	100.0		
	1 hr.		1,536	109.0	696	102.0	88.0	100.0	1,960	107.0		
	5 hrs.		1,510	108.0	665	97.0	87.0	99.0	1,890	103.0		
7. Male mongrel; weight 16½ kilos.	0	No exercise on this day.	998	100.0	723	100.0	114.0	100.0	1,965	100.0		
	1 hr.		1,020	102.0	620	86.0	105.0	92.2	1,722	88.0		
	5 hrs.		985	100.0	613	85.0	106.0	93.0	1,705	87.0		



TEXT-FIG. 2.

TEXT-FIG. 2. Control findings. The animals were confined, throughout, to small cages.



TEXT-FIG. 3.

— Exercise series.
 - - - Control series.

of dye solution injected. Cell volume shows a marked decrease in four animals, a slight increase in one, and a considerable increase in another. The average for the series is a decrease of 3.2 per cent. Hemoglobin and pigment volume show irregular changes mostly within rather narrow limits. The red count also decreases slightly. The third set of determinations shows irregular variations in all the blood components but within narrow limits, and the average is very close to the normal, taking the results of the first determination as such.

In Table IV, the changes in both series of animals have been compared. Only the average variations in per cent of the initial or "normal" average are there given. These percentage changes are also made the basis of the graphic representation in Text-fig. 3 where

TABLE IV.
Summary of the Average Changes in the Two Series.

Determination.	Plasma volume.		Cell volume.		Hemoglobin.		Pigment volume.		Red corpuscles per c. mm.	
	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.	Exercise series.	Control series.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
First.	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Second.	103.6	104.6	112.3	96.8	108.5	98.6	116.1	99.2	111.0	97.1
Third.	111.7	101.6	109.4	99.0	99.6	98.8	108.4	98.9	92.6	104.5

the curves of the average changes in the two series are superimposed. The comparison demonstrates an initial increase of cell volume and of circulating blood pigment as a result of brief exercise. There is a slight initial increase in plasma volume. During more prolonged exercise the cell volume and circulating blood pigment tend to decrease while a dilution of the blood occurs by increase in plasma volume.

DISCUSSION.

The Plasma Changes.

Previous studies by other workers have shown that considerable reliance may be placed in plasma volume figures obtained by the vital red method when properly carried out. Our findings indicate that

the initial concentration of the blood during exercise is not due to a loss of fluid, since there is no decrease in plasma volume. On the contrary, as stated by Cohnheim⁴ and others of his school, there is a distinct increase, especially when exercise is prolonged. As to the mechanism of this increase, Gross and Kestner⁵ and Cohn¹⁹ have offered considerable evidence to show that the fluid is taken from the muscles. They believe in a close connection between the increase in plasma volume and profuse excretion of sweat, but our results were obtained in dogs, animals which do not sweat in the ordinary sense. However, water is lost by them in considerable quantities in the process of panting.

The Alterations in the Distribution of Red Cells.

The calculation of total cell volume was made on the basis of the percentage volume of cells in the jugular vein blood, the total plasma volume being known. Hemoglobin, pigment volume, and red count are also influenced by changes in the ratio of cells and plasma. The initial increase in total cell volume shown in these experiments cannot have been due to a mere swelling of the cells, since the hemoglobin increased also. It might conceivably result either from a production of new blood corpuscles or a redistribution of those already in the body. The rapidity of the reaction definitely excludes the first possibility. Schneider³ has called attention to the similarity of the cell changes during exercise to those taking place in the early stages of acclimatization to high altitudes, and he believed that a reserve supply of corpuscles from the abdominal vessels is thrown into the peripheral blood stream in both instances. Campbell and Hoagland²⁰ have found that during the process of acclimatization to high altitudes, the peripheral blood contains cells in greater concentration than the mesenteric blood. It is therefore quite possible that, under normal resting conditions, the peripheral vessels are less rich in corpuscles and contain a greater per cent of plasma than the abdominal, and that in response to the demands of exercise, a redistribution takes place.

¹⁹ Cohn, E., *Z. Biol.*, 1920, lxx, 366.

²⁰ Campbell, W. A., and Hoagland, H. W., *Am. J. Med. Sc.*, 1901, cxxii, 654.

If we accept the view of Schneider,³ one can readily explain how it was that Cohnheim and his coworkers⁴ found a decrease in per cent of hemoglobin after the prolonged exertion of mountain climbing. They were at high altitudes before starting on these expeditions, and therefore the readjustments due to acclimatization had already increased as far as possible the per cent of cells in the peripheral blood. The increase in plasma volume accompanying exercise would therefore lead at once to dilution of the blood and the fall in per cent of hemoglobin which they actually observed.

The Changes in the Size of Red Corpuscles.

It is not impossible that changes in the size of red cells occur during exercise. A general swelling of the cells could readily lead to an increase in cell volume. However, the close parallelism between the curves of cell volume and pigment volume in the present work indicates that the results are not due to this cause—for were this the cause there would be disproportionate changes in pigment content and cell bulk. The red counts here recorded are too few in number to furnish reliable data as to average cell size. The literature on changes in cell size during exercise is extremely meager. Price-Jones,²¹ working with dried blood smears, has concluded that the cells become larger. The present findings would, in general, tend to confirm this view, for the decrease in cell volume during prolonged exercise is not nearly so great as the decrease in pigment volume. One might account for this by a disappearance of some cells from the circulation and an increase in size of those that remain.

Evidences of Blood Destruction.

It will be noted that after prolonged exercise both total cell volume and pigment volume fall well below the maximum noted after 10 minutes of exercise. The decrease observed in percentage of hemoglobin and in the number of cells per cubic millimeter is less significant, being partly due to dilution. In view of the uncertain factor of cell distribution, it would be unsafe to conclude that blood de-

²¹ Price-Jones, C., *J. Path. and Bact.*, 1919-20. xxiii, 371.

struction occurs during exercise on the basis of the present data alone, although there is no doubt that they are in harmony with such a view. More facts in the matter will be given in later papers.

SUMMARY.

The following changes have been demonstrated to take place in the blood of dogs during exercise.

1. An increase in the per cent of cells and hemoglobin in the blood of the jugular vein occurs early in the course of exercise. It probably results from a redistribution of red corpuscles, with an increase in their proportion in the peripheral blood.

2. As exercise is continued, there is a definite increase in plasma volume.

3. A coincident decrease both in the total cell volume and the pigment volume during prolonged exercise suggests that blood destruction then occurs.

BLOOD DESTRUCTION DURING EXERCISE.

II. DEMONSTRATION OF BLOOD DESTRUCTION IN ANIMALS EXERCISED AFTER PROLONGED CONFINEMENT.

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(Received for publication, August 7, 1922.)

In a previous communication¹ it was shown that a slight decrease in the total red cell volume and hemoglobin content of the organism frequently occurs during a single day of exercise in dogs previously kept under sedentary conditions. The decrease, however, is of very small proportions and not invariable, so it scarcely yields convincing proof of an increase in blood destruction under the circumstances mentioned. Observations on the effects of several consecutive days of exercise are obviously called for.

Nothing is more certain than that under the varied conditions of normal life blood formation keeps exact pace with blood destruction. If blood destruction is relatively greater during exercise, then assuredly the marrow must put forth more cells in animals habituated to exercise. Otherwise anemia would develop, and this is not observed. But, as is well known, stimulation of the marrow, even from considerable blood losses by hemorrhage, is not effective at once. Only after several days does the replacement of lost corpuscles go on actively. In the experiments to be described this circumstance has been utilized. Animals long accustomed to a sedentary life, and in which presumably little blood was being destroyed or made, were exercised vigorously with the idea that the blood-forming tissue might be "caught napping" and not at once make up such unusual corpuscle losses as exercise would entail.

Experimental Procedures.

Both the carbon monoxide and the dye methods for blood volume determination were employed.

¹ Broun, G. O., *J. Exp. Med.*, 1922, xxxvi, 481.

The technique of the dye method has already been described.¹ In that with carbon monoxide the procedure of Van Slyke and Salvesen² was used in the blood gas analysis. The CO readings were checked by absorbing the gas with cuprous chloride solution. The arrangement of the respiratory chamber resembled that described by Arnold and his coworkers.³ The CO, however, was introduced directly into the base of the metal cone that closely covered the nose and mouth of the animal. The determination of residual CO in the respiratory chamber was likewise made by the method which Arnold employed.

Dogs were used, as in the preceding study, and only such as had been kept caged for several months. All were healthy adults and, with one exception, males. They were fed upon a generous mixed diet containing considerable meat. No change was made in the amount or quality of the food during the period of observation. On the days on which the animals were exercised, they were fed after its conclusion, since when food was given before or during exercise vomiting was frequent. Treadmills were employed with the tread at an angle of 20° to the horizontal. The average day of exercise consisted of two 2 hour periods of treadmill work separated by a rest period of 1 hour. The dogs worked willingly and, especially in the first few days, were very tired when released. Water was allowed in the rest interval. Each dog was given 3 to 6 consecutive days of exercise. All ate well and remained in excellent health and spirits, towards the end of the 6 days appearing to take pleasure in the exercise.

The number of preliminary blood volume determinations and the intervals between them were intentionally varied in order to rule out the possibility that the determinations themselves were responsible for such changes as occurred. The control observations vary, therefore, in number from one to five, and the preliminary observation periods from a single day to 3 weeks. The control periods of some animals overlap the exercise periods of others of the same series and hence one can be certain that no common factor of intercurrent nature was active in depressing the cell volume. It did not seem wise to use the dye and the carbon monoxide methods together, on the same individual, since the double procedure is taxing. A separate series of animals was therefore used for each type of determination. The observations with the CO method were made during the winter months, those with the dye method during the spring and summer, so no seasonal factor can be invoked to explain the parallelism in result.

In some instances a determination of blood volume was made during the days of exercise. In all cases a determination was made on the day on which exercise was discontinued. Five out of six of the animals of Series A (Table I) were kept under observation until the cell volume returned to normal levels, or went above, as sometimes happened. Only occasionally was this done with the animals of the other two series (Tables II and III).

¹ Van Slyke, D. D., and Salvesen, H. A., *J. Biol. Chem.*, 1919, xl, 103.

² Arnold, H. R., Carrier, E. B., Smith, H. P., and Whipple, G. H., *Am. J. Physiol.*, 1921, lvi, 313.

The average of such cell and plasma volume determinations as were made prior to exercise furnished the "normal" in each instance. In the tables all determinations are given both as the actual number of cubic centimeters of cells or plasma and as percentages of this normal, taking the latter as 100 per cent. The percentage figures are made the basis of the graphic representation in the charts.

Results by the Carbon Monoxide Method.

The findings in the six animals of Series A are given in Table I and Text-fig. 1. In each instance the blood volume was determined by the CO method. It will be seen that all the animals showed a decrease in cell volume during the exercise period. The average decrease for the series was 19 per cent. The changes in plasma quantity were very irregular; but the CO method is known to be ill suited for plasma volume determinations,⁴ so no significance pertains to the variations observed.

Results by the Dye Method.

Two series of animals were exercised, and the changes in blood volume followed by the dye method.

In Series B (Table II, Text-fig. 2) the blood volume determinations during the preliminary or control period were made in the absence of any exercise, so far as this could be avoided in animals as lively as dogs; while, with one exception (Dog 6), 18 hours of rest were allowed to elapse after exercise before the later determinations, in order that the distribution of cells, natural during rest, might reestablish itself. In Series C (Table III, Text-fig. 3) every determination was immediately preceded by exercise of at least 10 minutes duration, to secure the altered distribution of cells which brief activity brings about. As has already been shown¹ a false increase in cell volume consequent on changed cell distribution occurs during brief exercise when the determinations are made by the dye method.

In all of the animals a distinct fall in cell volume was noted. For Series B the average decrease amounted to 25 per cent; for Series C 18 per cent. The agreement with the findings by the CO method is

⁴ Smith, H. P., Arnold, H. R., and Whipple, G. H., *Am. J. Physiol.*, 1921, lvi, 336.

TABLE I.
Series A. Blood Destruction during Exercise as Shown by the CO Method.

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 0. Male mongrel; weight 16½ kilos.	4 days before beginning exercise.	670	96	960	101	Animal caged 5 mos. at the time observations were begun.
	1 day " "	723	104	939	99	No exercise. " Exercise begun the next day.
	After 4 days of exercise.	591	85	919	97	Animal exercised 4 hrs. daily for 4 days.
	19 days later.	677	97	825	87	Blood volume determination at end of exercise period.
	5 " after preceding determination.	710	102	672	71	No exercise since preceding determination.
No. 1. Male collie; weight 22 kilos.	6 " before beginning exercise.	1,165	94	1,090	104	Animal caged 3 mos. at the time observations were begun.
	4 " " "	1,270	103	975	93	No exercise. " "
	The day exercise was begun.	1,276	103	1,074	103	" " before blood volume determination.
						Exercised 2 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days.

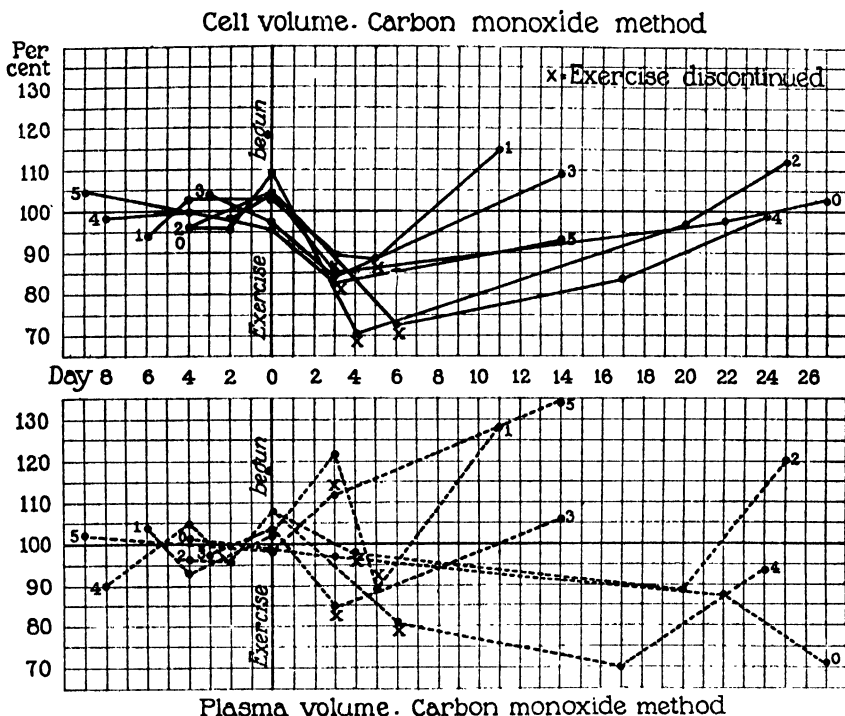
No. 2. Male mongrel; weight 14½ kilos.	After 4 days of exercise.	1,096	89	1,272	122	Blood volume determination at the end of exercise period.
	" 6 " "	1,089	88	936	89	Blood volume determination at the end of exercise period.
	6 days after preceding determination.	1,425	115	1,335	128	No exercise since preceding determination.
	4 " before beginning exercise.	686	96	714	96	Animal caged 5 mos. at the time observations were begun.
	2 " " "	682	95	710	96	No exercise.
	The day exercise was begun.	778	109	802	108	" " before blood volume determination. Exercised 4 hrs. on this day and 4 hrs. daily for the next 4 days.
	After 5 days of exercise.	504	70	726	98	Blood volume determination at end of exercise period.
	16 days after preceding determination.	694	97	658	89	No exercise since preceding determination.
	5 " " "	800	112	888	120	No exercise since preceding determination.
						Animal caged 5 mos. at the time observations were begun.
No. 3. Male pointer; weight 20½ kilos.	3 " before beginning exercise.	825	104	1,110	97	No exercise.
	The day exercise was begun.	767	96	1,180	103	" " before blood volume determination. Exercised 4 hrs. after blood volume determination and 4 hrs. daily for the next 3 days.
	After 4 days of exercise.	669	84	968	85	Blood volume determination at end of exercise period.
	11 days after preceding determination.	866	109	1,206	105	No exercise since preceding determination.

TABLE I—*Concluded.*

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 4. Female collie; weight 23½ kilos.	10 days before beginning exercise.	1,128	98	1,084	90	Animal caged 5 mos. at the time observations were begun.
	6 " "	1,148	100	1,267	105	No exercise.
	4 " "	1,130	98	1,160	96	" "
	2 " "	1,195	104	1,305	108	" " 2 days later exercise was begun and the animal exercised 4 hrs. daily for 5 days.
	After 5 days of exercise.	834	73	978	81	Blood volume determination at end of exercise period.
	11 days after preceding determination.	960	83	840	70	No exercise since preceding determination.
	7 " "	1,143	99	1,127	94	No exercise since preceding determination.
No. 5. Male collie; weight 20 kilos.						Animal caged 2 mos. at the time observations were begun.
	9 " before beginning exercise.	1,081	103	1,043	102	No exercise.
	The day exercise was begun.	1,010	97	1,010	98	" " before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 3 days.
	After 4 days of exercise.	867	83	1,145	112	Blood volume determination at end of exercise period.
	11 days after preceding determination.	972	93	1,373	134	No exercise since preceding determination.

therefore striking. Since the factor of cell distribution was controlled as already indicated, the findings point to an actual decrease in the volume of circulating cells.

As one would expect, the plasma volume curves obtained by the dye method are much more regular than those from the CO determinations. In the majority of instances, an increase in plasma was



TEXT-FIG. 1. Series A. Blood destruction during exercise as shown by the CO method. Exercise was begun 2 days after the point indicated in Dog 4.

found at the end of the days of exercise. Such was the case in eight of the eleven experiments recorded (Tables II and III). In two animals (Nos. 8 and 9, Table II, Text-fig. 2), although a slight decrease occurred from the plasma volume noted at the beginning of exercise, the figures are still above the normal average. A distinct decrease below the normal took place only during the first experiment in Dog 7 (Table II, Text-fig. 2). This increase in plasma, occurring as it

TABLE II.
Series B. Blood Destruction during Exercise as Shown by the Dye Method (Preliminary Observations Carried Out with the Animal Quiet).

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 7. Male pointer; weight 18 kilos.	2 days before beginning exercise. The day exercise was begun.	1,003 984	101 99	1,212 1,346	95 105	Animal caged 3 mos. at the time observations were begun. No exercise. " " before blood volume determination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 2 days. Animal rested 18 hrs. before blood volume determination. No exercise since preceding determination.
	After 3 days of exercise.	620	62	1,160	91	
	7 days after preceding determination.	760	77	1,300	102	
	14 " before beginning exercise. 4 " " 3 " " The day exercise was begun.	532 548 511 577	98 101 94 106	706 698 759 795	96 95 103 108	Animal caged 3 mos. at the time observations were begun. No exercise. " " " " before blood volume determination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 3 days. Animal rested 18 hrs. before blood volume determination.
No. 8. Male pointer; weight 14½ kilos.	After 4 days of exercise.	451	83	763	103	

No. 9. Male collie; weight 14 kilos.	21 days before beginning exercise.	415	99	878	100	Animal caged 3 mos. at the time of variations were begun. No exercise. " " " " " " " " " " " " before blood volume deter- mination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days. Animal rested 18 hrs. before this deter- mination. No exercise since preceding deter- mination. Animal caged 4 mos. at the time obser- vations were begun. No exercise. " " " " " " " " " " before blood volume deter- mination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days. Blood volume determination at end of exercise period. Exercised 2 hrs. the next day. Exercise then dis- continued. No exercise.
	18 " " "	390	93	818	93	
	14 " " "	406	97	839	96	
	3 " " "	420	100	900	103	
	The day exercise was begun.	469	112	957	109	
	After 5 days of exercise.	312	74	916	104	
	3 days after preceding determination.	352	84	918	105	
	9 " before beginning exercise.	923	103	1,062	91	
	6 " " "	902	101	1,150	99	
	4 " " "	849	95	1,136	98	
No. 6. Male pointer; weight 20½ kilos.	2 " " "	873	97	1,212	104	Animal caged 4 mos. at the time obser- vations were begun. No exercise. " " " " " " " " " " before blood volume deter- mination. Exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days. Blood volume determination at end of exercise period. Exercised 2 hrs. the next day. Exercise then dis- continued. No exercise.
	The day exercise was begun.	936	104	1,244	107	
	After 5 days of exercise.	737	82	1,295	112	
	3 days after preceding determination.	911	102	1,164	100	

TABLE III.
Series C. Blood Destruction during Exercise as Shown by the Dye Method (Preliminary Determinations Preceded by Brief Activity).

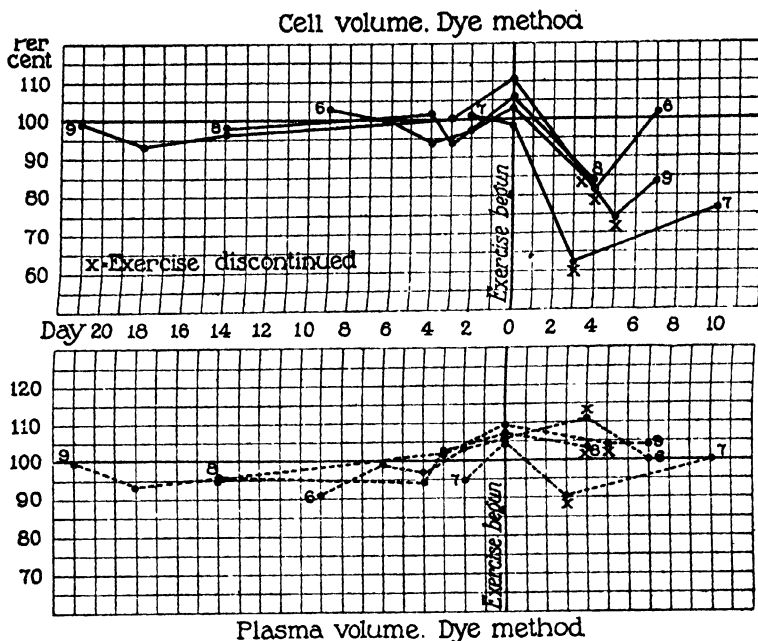
Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 7. Male pointer; weight 18 kilos.	The day exercise was begun.	990	100	1,133	100	Animal caged 1 mo. without exercise since observations recorded in Text-fig. 2 were made. Cell volume has returned to normal level. 10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days. Blood volume determination at end of exercise period. Blood volume determination at end of exercise period. Animal rested 1 day. Exercised 1 hr. daily on the 2 following days. 1 hr. of exercise before blood volume determination. Animal rested 1 day. Exercised 1 hr. daily for 3 days and again rested 1 day. 10 min. exercise before blood volume determination.
	After 2 days of exercise.	960	97	1,330	117	
	" 6 " "	811	82	1,177	104	
	3 days later.	820	83	1,290	114	
	6 days after last determination.	926	94	1,279	113	

No. 10. Male mongrel; weight 14 kilos.	The day exercise was begun.	618	100	762	100	Animal caged 4 mos. at the time observations were begun. 10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 2 days.
	After 1 day of exercise.	589	95	785	103	No exercise before this blood volume determination.
	" 2 days "	490	79	795	104	4 hrs. exercise before this blood volume determination.
	" 3 " "	483	78	793	104	15 min. exercise before this blood volume determination.
	6 days before beginning exercise.	1,025	105	990	100	Animal caged 2 mos. at the time observations were begun. 10 min. exercise before blood volume determination. Otherwise no exercise.
No. 11. Male setter; weight 19½ kilos.	4 " " "	940	96	1,032	105	10 min. exercise before blood volume determination. Otherwise no exercise.
	The day exercise was begun.	970	99	935	95	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days.
	After 4 days of exercise.	754	77	998	101	4 hrs. of exercise before blood volume determination.
	" 6 " "	755	77	992	101	4 hrs. of exercise before blood volume determination.
	4 days after preceding determination.	804	82	1,038	105	Animal rested 1 day and then exercised 1 hr. daily for 3 days. 10 min. exercise before blood volume determination. Otherwise no exercise.

TABLE III—Continued.

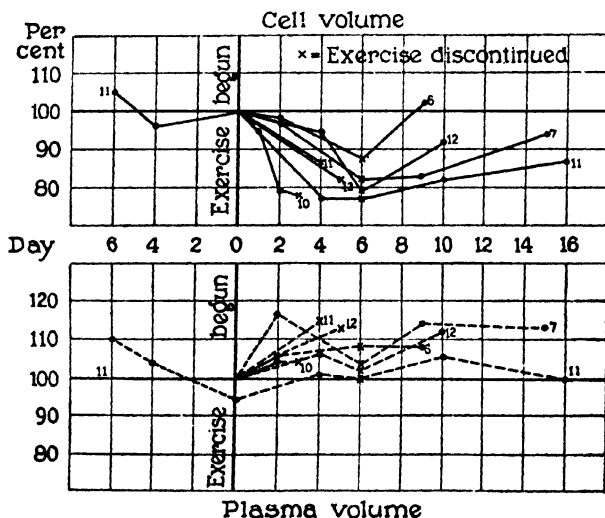
Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 11—continued.	6 days after preceding determination.	848	87	990	100	10 min. exercise before blood volume determination. Otherwise no exercise.
No. 11. Male setter; weight 19½ kilos.	The day exercise was begun.	863	100	918	100	Animal caged 40 days without exercise since observations noted above.
	After 4 days of exercise.	740	86	1,052	115	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 3 days.
No. 12. Male mongrel; weight 18½ kilos.						10 min. exercise before blood volume determination.
	The day exercise was begun.	940	100	912	100	Animal caged 5 mos. at the time observations were begun.
	After 4 days of exercise.	887	94	968	106	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days.
	" 6 " "	740	79	935	102	4 hrs. exercise before blood volume determination. 4 hrs. exercise before blood volume determination. Animal rested 1 day and then exercised 1 hr. daily for 2 days.

	4 days after preceding determination.	866	92	1,024	112	15 min. exercise before blood volume determination.
No. 12. Male mongrel; weight 16½ kilos.						Animal caged 45 days without exercise since observations noted above. Animal has lost weight and has smaller blood volume.
	The day exercise was begun.	760	100	848	100	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 4 days.
	After 5 days of exercise.	624	82	948	113	Blood volume determination made after 4 hrs. of exercise.
No. 6. Male pointer; weight 20½ kilos.						Animal exercised 6 days during preceding week. Rested a day and a half. Cell volume has returned to normal level (see No. 6, Text-fig. 2 and Table II).
	The day exercise was begun.	911	100	1,164	100	10 min. exercise before blood volume determination. Animal exercised 4 hrs. on this day after blood volume determination and 4 hrs. daily for the next 5 days.
	After 2 days of exercise.	891	98	1,233	106	Blood volume determination at end of exercise period.
	" 6 " "	797	87	1,253	108	Blood volume determination at end of exercise period.
	3 days later.	930	102	1,262	108	Exercise discontinued. 1 hr. of exercise before blood volume determination.



TEXT-FIG. 2. Series B. Blood destruction during exercise as shown by the dye method. The preliminary observations were carried out with the animal quiet.

Dye method preceded by exercise



TEXT-FIG. 3. Series C. Blood destruction during exercise as shown by the dye method. The preliminary determinations were preceded by brief activity.

did simultaneously with a decrease in cell volume, may represent an effort to maintain a constant total blood volume. On the other hand, evidence has already been given¹ that as little as several hours of exercise on a single day cause an increase in plasma. It is significant that the three determinations that show a decrease in plasma to have been present at the end of the exercise were made, not immediately thereafter, but when 18 hours of rest had elapsed.

The Period of Recovery.

The time required for the cell volume to return to normal levels after the depression due to exercise is subject to wide variations. All but one of the animals of Series A (Table I, Text-fig. 1) were kept in their cages until recovery was complete. Dog 1 recovered completely during the 1st week after exercise was discontinued; No. 3 in the course of the 2nd week; while Nos. 0, 2, and 4 did not reach normal levels until nearly 3 weeks had elapsed.

Nos. 1, 2, and 3 later developed a well marked plethora; No. 1 yielding the highest cell volume per kilo that the writer has encountered in supposedly normal dogs. No less than three blood volume determinations were made on the animal at this time, two by the CO method and one by the dye method. All gave practically the same results. Only the first of these three determinations is recorded in Text-fig. 1 and Table I. The others find place in a subsequent paper.⁵ Dog 5 of the series was utilized for other purposes after the 11th day following exercise. At this time his cell volume was 93 per cent of the normal.

In Series B (Table II, Text-fig. 2) and Series C (Table III, Text-fig. 3) the course of recovery was seldom followed to its close. Dog 6 recovered within 3 days after the initial period of exercise (Table II, Text-fig. 2). Further exercise during the following week (Table III, Text-fig. 3) caused a second decrease in cell volume, repaired by the animal with equal rapidity. No. 7 (Table II, Text-fig. 2) showed but slight tendency to recovery 1 week after the first exercise period, but 1 month later the cell volume was normal (Table III, Text-fig. 3). A second period of exercise by this animal was followed

⁵ Brown, G. O., *J. Exp. Med.*, 1923, xxxvii (in press).

by a more rapid recovery,—the cell volume coming up to 94 per cent in the 1st week. The cell volume of Dog 11 (Table III, Text-fig. 3) reached 87 per cent of the normal within 10 days after the 1st week of exercise. 1 hour of exercise was given on 3 days of this interval. 40 days later the cell bulk was still far below the normal, as is seen from the preliminary determinations for the second experiment. No. 12 (Table III, Text-fig. 3) under similar conditions had, within 4 days after the first exercise period, a cell volume that amounted to 92 per cent of the normal. A month and a half later a very small cell volume was found, one practically of the same proportions noted at the end of the first exercise period. The body weight had decreased by 2 kilos but the animal seemed in excellent health and spirits and remained in good condition for several months thereafter. A second period of exercise caused the cell volume of both Dogs 11 and 12 to fall below the new level that had been established (Table III, Text-fig. 3).

Control of the Nutritive Factor.

As has already been stated, food in plenty was provided and the amount taken was frequently weighed. The appetite of the animals

TABLE IV.

Control of the Nutritive Factor.

Animal.	Time.	Cell volume.		Plasma volume.		Remarks.
		Cc.	Per cent of normal average.	Cc.	Per cent of normal average.	
No. 25. Female collie; weight 22½ kilos.	The day observation was begun.	1,148	100	1,026	100	Food withheld 4 days.
	4 days later.	1,144	100	1,028	100	
No. 26. Male pointer; weight 17 kilos.	The day observation was begun.	855	100	1,038	100	Food withheld 4 days.
	4 days later.		102	1,061	102	

Determinations were made by the CO method.

in no instance suffered from the exercise, yet as a control to possible underfeeding during the exercise period, the following experiment was performed.

Food was entirely withheld from two animals during a period of 4 days. Blood volume determinations were made at the beginning and end of this period.

The results are given in Table IV. It will be seen that no decrease in cell volume occurred.

DISCUSSION.

The fact cannot be too clearly emphasized that the decreases in circulating hemoglobin and total cell volume which were caused by the exercise employed in the present work occurred in animals previously kept in confinement for several months. Experiments, which will be described in detail in a later communication,⁵ show that in dogs allowed liberty or previously exercised during a considerable period of time, the cell volume either fails to decrease when exercise is given according to the method described or shows but slight decrease.

Most of the literature on the effect of exercise on blood has to do with the changes occurring in the course of a single day of exercise. It has already been considered.¹ Schneider and Havens⁶ followed the red count and hemoglobin on three athletes at intervals during a period of training, and noted slight increases in both at the end of the period. The blood volume was followed on a single subject, several observations being made, before the training period and after training had been in progress for more than 3 weeks. No marked changes occurred. These findings are not contradictory to the ones here reported. The athlete studied had doubtless led an active life prior to training. Moreover, in the space of 3 weeks of training the hematopoietic system may completely adapt itself to the demands of exercise. Feigl⁷ has shown that after strenuous marches many individuals show traces of hemoglobin and hematin in the blood serum and urine—evidence of injury to the blood.

Whipple⁸ has reported that carbon monoxide is to some extent taken up by the myohematin of the muscles. It may be asked whether

⁶ Schneider, E. C., and Havens, L. C., *Am. J. Physiol.*, 1914-15, xxxvi, 239.

⁷ Feigl, J., *Biochem. Z.*, 1916, lxxvi, 88.

⁸ Whipple, G. H., *The Harvey Lectures*, 1922 (in press).

the changes in cell volume shown by the CO method are referable in any part to this factor. For this to be so a marked decrease in myohematin must occur during exercise. But similar changes in cell volume are demonstrable with the dye method and the dye employed in the work, vital red, is not taken up by the respiratory pigments. It follows that the changes in cell volume are dependent on actual changes in the amount of circulating cells. It is possible that the high volume figures found by the CO method during the period of recovery may be due in some small part to an increase in myohematin consequent on hypertrophy of the muscles, though there is abundant precedent for these figures in the plethora that ordinarily follows recovery from the experimental anemias.

SUMMARY.

A great decrease in total circulating hemoglobin and red cell volume occurs in dogs long maintained under sedentary conditions when they are exercised vigorously during several consecutive days. This would appear to be consequent on increased blood destruction, unrepaired for the time being.

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IX. THE RECURRENCE OF 1922.

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PLATES 46 TO 49.

(Received for publication, June 27, 1922.)

INTRODUCTION.

The presence in New York City during the early months of 1922 of an acute respiratory infection that, clinically, resembled more or less the epidemic influenza of the autumn and winter of 1918-19 and the spring of 1920 afforded us an opportunity to repeat and extend the studies of the nasopharyngeal secretions from influenza patients which had resulted in the isolation of *Bacterium pneumosintes*. The reports of the earlier experiments have formed Papers I to VIII of the present series.¹⁻⁸

As an introduction to our observations with material obtained during the 1922 recurrence of influenza, the facts already established may be passed briefly in review, since they form the basis for a comparison of the data recently obtained with the findings in similar experiments during the past 3½ years.

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

² Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

⁴ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

⁵ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

⁶ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

⁷ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.

Summary of Earlier Observations.

Having in mind the possible presence in the nasopharyngeal secretions of influenza patients of an agent whose effects might be noted in animals, we injected the whole or the filtered nasopharyngeal washings intratracheally into rabbits. In eight instances the nasopharyngeal washings were obtained within 36 hours of the onset of the uncomplicated disease. The material from five of these patients during the epidemic wave of 1918-19 and from two during the recurrence of 1920 produced typical clinical and pathological effects in the experimental animals. On the other hand, the active agent was not obtained from twelve influenza patients, the onset of whose illness had occurred more than 36 hours previously, nor from fourteen persons free from influenza during the epidemic and interepidemic periods.¹

The effects produced by the active agent in the experimentally infected animals bore a certain similarity to those observed in epidemic influenza in man. Clinically, the affection was typified by a rise in temperature within 24 to 48 hours, by conjunctivitis, and by a characteristic change in the blood picture—a rapidly developing leucopenia, due mainly to a critical drop in the mononuclear cells. Unless the train of events was interrupted by secondary infection, the rabbits returned to normal in the course of 2 or 3 days. Most of them were killed after 24 to 48 hours, however, for observation of the lung lesions and to obtain material for further passage, and for cultivation. In such instances a typical pathological condition was revealed, consisting of a diffuse hemorrhagic edema of the lungs, with emphysema, and with a peculiar interalveolar cellular exudate, and a similar hemorrhagic involvement of bronchi and trachea, with exfoliation of the necrotic epithelium.¹

When lung tissue containing such lesions was ground and extracted with salt solution, the turbid suspension produced similar effects in other rabbits, and in this way the substance active in the process was transmitted for as many as fifteen passages, indicating the multiplication of a living agent rather than the mere passive transfer of some original material.

This living agent was found to have the following properties.

As it existed in the nasopharyngeal secretions in man, and in the lungs of affected rabbits, it passed through Berkefeld V and N candles, and the filtered material produced the same effects on the blood and in the lungs of rabbits and guinea pigs as the unfiltered material.²

When contained in bits of lung tissue, it withstood the action of a 50 per cent glycerol solution for periods up to 9 months.²

It produced effects upon the pulmonary tissues of rabbits which favored the invasion and infection of the lung with other bacteria, such as the pneumococcus, streptococcus, and *B. Pfeifferi*.³

It led to the development, in experimentally infected animals, of a specific immunity against reinfection with similar material.⁶

Meanwhile, cultivation experiments had shown the presence in the active material of a hitherto undescribed microorganism. This microorganism, which was subsequently named *Bacterium pneumosintes* because of its injurious effect upon the lung, was first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Subsequently the same bacterium was isolated from material originally derived from all the seven cases of influenza from which active material was transmitted to rabbits, and from three other cases not so transmitted. The immediate sources of the cultures were the filtered nasopharyngeal secretions of patients or, more frequently, the unfiltered or filtered lung tissue suspensions of affected rabbits. These rabbits had been intratracheally injected with whole or filtered nasopharyngeal secretions, whole or filtered lung suspensions of other affected animals, or the glycerolated lung tissue of such animals. The cultivation experiments were carefully controlled with similar materials from non-influenzal sources. All of the control materials gave negative results.⁴

The primary cultures of *Bacterium pneumosintes* were obtained under strictly anaerobic conditions in a medium composed of sterile human ascitic fluid and a fragment of fresh rabbit kidney. In this medium the microorganism developed slowly, producing a faint haze in the region of the kidney fragment that gradually extended to a depth of about 3 cm. by the 8th day and in the course of 2 weeks settled to the bottom of the tube, leaving a clear, faintly opalescent, supernatant fluid.

In stained films, *Bacterium pneumosintes* appeared as a minute bacilloid body of regular morphology, measuring 0.15 to 0.3 micron in the long axis. Usually solitary, the bacteria were often found in diplo form, and occasionally in short chains of three or four members. All of the strains decolorized uniformly by Gram's method. They were stained with some difficulty by the usual basic dyes.

The intratracheal injection, in rabbits and guinea pigs, of mass cultures of *Bacterium pneumosintes* in the earlier generations induced effects on the blood and lungs of these animals which could not be distinguished from those obtained with the active agent of the nasopharyngeal secretions of influenza patients. In addition to this identity of source and of pathogenic effect in animals, *Bacterium pneumosintes* exhibited the other qualities characteristic of the active agent. Thus the microorganism passed Berkefeld V and N filters even in remote generations. Protected by the lung tissue of rabbits affected by the injection of mass cultures, it withstood glycerolation for a period of 9 months. Infection with *Bacterium pneumosintes* reduced the resistance of the pulmonary tissues of rabbits to infection with other bacteria, such as *Pneumococcus* Type IV and *B. Pfeifferi*.⁴ Finally, by cross-immunity experiments, the antigenic identity of the various strains of *Bacterium pneumosintes* with each other and with the active influenzal agent was completely established.

Before methods had been found for the cultivation of *Bacterium pneumosintes* in simplified media, the opportunity was lost to examine for specific antibodies the blood serum of persons convalescent from influenza or of most of the animals

which had recovered from the experimental infection. Later, methods of cultivation were devised which did not interfere with the detection of specific antibodies.^{8,9} Rabbits intravenously injected with such cultures produced antibodies which were recognized by precipitation, agglutination, complement fixation, and phagocytic tests, and animals injected with glycerolated lung tissue containing the active agent yielded serum that specifically agglutinated *Bacterium pneumosintes*. It is significant that the four available strains of *Bacterium pneumosintes*, three from 1918-19 and one from 1920, showed identical antigenic characters by serological and immunological tests.

More recent experiments on methods of cultivation⁸ have shown that *Bacterium pneumosintes* can be grown on media consisting of meat infusion peptone broth, or nutrient agar, as a base, enriched with fresh animal or vegetable tissue, fresh defibrinated rabbit blood, or by the growth of other organisms. Surface colonies have been obtained on blood agar plates in the Brown anaerobic jar.¹⁰ The plates have proved especially useful for the purification of contaminated cultures and for the demonstration of viable organisms in sparse primary growths in the ascitic fluid-rabbit kidney medium. When grown on media containing nutrient broth, and especially in the presence of dextrose, *Bacterium pneumosintes* has developed larger bacillary forms up to 1 micron in length. The identity of these microorganisms with the original strains has been proved by serological reactions and by their reversion to the minute forms on transfer to the original medium.

Animal Transmission and Cultural Studies with Recent Materials.

As a result of the experiments outlined above, we had facilities for animal transmission and cultural studies at hand during the recent outbreak of clinical influenza in January and February of the present year and were able to proceed without the loss of time that was formerly spent in an empiric search for methods.

This outbreak of influenza enabled us to examine the nasopharyngeal secretions of nine persons who had within a few hours developed symptoms characteristic of the disease. The following protocol is cited as a typical example of the cases studied.

Case 36.—Adult male. Feb. 9, 1922. Illness began at night with chilly sensations, a thin, clear secretion from the nose, fever, and pains in the joints and muscles, especially those of the back and legs. Feb. 10. Remained in bed. Temperature 38.3°C., marked coryza, intense injection of conjunctivæ, dry cough. Physical examination showed no pulmonary involvement. Leucocytes

⁸ Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.

¹⁰ Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

4,000, of which 3,920 were polymorphonuclear neutrophils, and 80 were mononuclears. On this day, 12 hours after onset, nasopharyngeal washings were obtained for examination. Feb. 11. Patient's condition improved. Feb. 12. Temperature normal. Recovery uneventful.

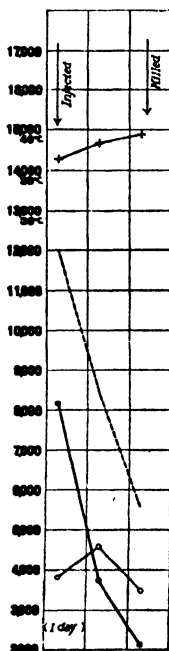
Eight other patients whose nasopharyngeal secretions were obtained from 6 to 36 hours after onset exhibited symptoms similar to those described. The washings from one of these patients stood at room temperature for 24 hours before transmission and cultivation experiments were undertaken. Both failed. Of the remaining eight specimens, successful transmission experiments were initiated with six. The seventh failed to induce the experimental disease in rabbits. The eighth specimen was not injected into animals, but after filtration through a tested Berkefeld candle it yielded a primary growth of *Bacterium pneumosintes* in an anaerobic ascitic fluid-rabbit kidney medium.

The following protocol of a typical animal transmission experiment is reported for comparison with the recorded results of the earlier experiments.¹

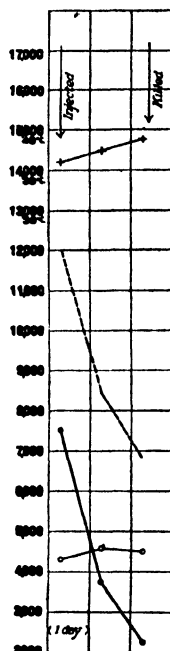
Case 28.—The unfiltered nasopharyngeal washings obtained 6 hours after onset were injected intratracheally into Rabbit A.

First Passage. Rabbit A (Text-Fig. 1).—Jan. 25, 1922. Leucocytes 12,000, of which 3,840 were polymorphonuclear cells and 8,160 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 2.5 cc. of unfiltered nasopharyngeal washings of Case 28. Jan. 26. Leucocytes 8,400, of which 3,780 were mononuclears. Temperature 39.7°C. Jan. 27. Leucocytes 5,600, of which 2,128 were mononuclears. Mild conjunctivitis. Temperature 39.9°C. Killed. All organs appeared normal except the lungs. These showed a diffuse edema and emphysema with a number of small hemorrhages scattered over the surface and on the cut sections. There was an absence of pneumonic consolidation. On microscopic examination (Fig. 1) the edema, emphysema, and the small hemorrhages were again revealed. There was a moderate degree of cellular infiltration of the interalveolar strands and about the bronchi and bronchioles, which consisted of mononuclears, polymorphonuclears containing acidophilic granules, and a few cells of the respiratory type. In addition, the interalveolar strands were distended with serum and erythrocytes. The bronchial system was also involved in a hemorrhagic and edematous process. The epithelium was necrotic in some areas and the lumen was filled with serum, leucocytes, erythrocytes, and exfoliated necrotic epithelial cells. Impression smears of the lung stained by Gram's method revealed numerous mononuclears and alveolar cells, but no bacteria. Aerobic cultures were free from growth.

In Giemsa-stained preparations of sections of the lung groups of minute organisms indistinguishable morphologically from *Bacterium pneumosintes* were noted in the bronchial exudate. Some were free and others in the different stages of phagocytosis by polymorphonuclears and monocytes (Fig. 2). In the ciliary margin of the bronchial epithelium similar clumps of these microorganisms were seen (Fig. 3).



TEXT-FIG. 1.



TEXT-FIG. 2.

- + — + Temperature.
- - - Total leucocytic count.
- — ● Mononuclear cells.
- — ○ Polymorphonuclear cells.

TEXT-FIG. 1. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. First rabbit passage of the nasopharyngeal washings from a patient (Case 28) in the early stage of uncomplicated epidemic influenza (1922). Intratracheal injection.

TEXT-FIG. 2. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. Rabbit passage of *Bacterium pneumosintes* from a culture, in the third generation, originally derived from a rabbit inoculated with the washings from another influenza patient (Case 36).

In the present series of experiments animal passage failed after the first transfer in one instance. The other transmission experiments were discontinued after the second to fourth transfers.

The effects produced by the intratracheal injection into rabbits of these specimens of unfiltered nasopharyngeal secretions from influenza patients appeared to us to be identical with those observed during the former epidemic waves. The active agent showed other familiar qualities also. It survived the action of 50 per cent glycerol for 4 months (the longest period yet tested) and passed Berkefeld V and N filters, producing characteristic lesions in rabbits injected with the filtrate.

As controls for these experiments, we have negative transmission experiments with the nasopharyngeal secretions of three persons not suffering from influenza. One of these had a mild head cold which ran a typical course; the others were normal.

Because of the identity of the active agent with *Bacterium pneumosintes* in former experiments, our endeavor was directed mainly toward the demonstration of a filterable anaerobic microorganism in the material obtained during this recurrence of influenza. Filtered nasopharyngeal secretions from each of the nine influenza patients and from ten other persons were inoculated into ascitic fluid and rabbit kidney tubes under a vaseline seal, and spread upon blood agar plates in an anaerobic jar. The lung tissues of affected rabbits were likewise cultured in the fluid medium, and sometimes smeared on the anaerobic plates. When no organisms or only a few minute and questionable bodies could be demonstrated with the microscope in fluid cultures, material from these cultures was spread on blood plates for the possible observation of colony formation.

By these methods there were obtained cultures of strictly anaerobic, filter-passing, Gram-negative organisms in material derived from four of the six influenza patients from whom an active agent had been transmitted to rabbits, from a seventh influenza patient whose nasopharyngeal washings were not injected into animals, and from four of ten other persons not suffering from influenza. A survey of these microorganisms showed that *Bacterium pneumosintes* is not the only anaerobic, filter-passing, Gram-negative organism to be found in the human respiratory tract. From one of the influenza patients and from

the four non-influenzal controls which yielded positive cultures, other bacteria, not pathogenic for rabbits and not *Bacterium pneumosintes*, were obtained. A preliminary description of these other bacteria is appended to this paper.

On the other hand, we have identified as *Bacterium pneumosintes* the cultures derived from three of the six influenza patients, enumerated above, whose nasopharyngeal secretions were pathogenic for rabbits, and from the seventh patient, whose secretions were not used in a transmission experiment. No cultures of *Bacterium pneumosintes* were obtained from the controls.

It is noteworthy that only one strain of *Bacterium pneumosintes* was obtained directly from the filtered nasal washings. The others were derived from lung tissue after rabbit passage. In this connection the relative value of these two immediate sources may be indicated. Nasopharyngeal secretions require filtration before direct cultivation in fluid media and much of the active material, mixed with mucus and epithelial debris, is retained on the filter and lost. It is probable that conditions are only occasionally favorable for the primary development of *Bacterium pneumosintes* in artificial culture. As with the pneumococcus a relatively large inoculum is usually required to initiate growth. Moreover, if the first inoculations fail the entire opportunity is lost. But when the whole nasopharyngeal washings are injected into rabbits, the larger primary inoculum is further augmented by active multiplication in the pulmonary tissues, and in those favorable instances in which contaminating bacteria are suppressed, a material rich in *Bacterium pneumosintes* in pure culture is available for artificial cultivation either directly or after filtration. Moreover, through continued rabbit passage repeated opportunities are afforded for cultivation experiments and sooner or later they usually succeed. We feel that although direct cultivation of filtered nasopharyngeal secretions may be a suitable method for the demonstration of other anaerobic filter-passing microorganisms, a negative experience in the cultivation of *Bacterium pneumosintes* without the aid of animal passage is of little significance.

Identification of New Strains of Bacterium pneumosintes.

The identification of the new strains of *Bacterium pneumosintes* was made on morphology, cultural characters, filterability, typical pathogenicity for rabbits, resistance to glycerol, reduction of resistance to secondary infection, and serological and immunological reactions.

Not all of these tests have been made with each strain, but each has been definitely identified by means of a combination of them, and all

have been agglutinated specifically by sera made with the 1918-19 strains as antigen. These experiments also served as a review and check on the characters of *Bacterium pneumosintes* as formerly observed, and confirmed our reported findings. For these reasons they may be described more or less in detail.

Morphological and Cultural Characters.—The morphology and cultural characters of the new strains are in all respects identical with those of the strains obtained in 1918-19 and 1920. Photographs of Strain 16,⁴ from the 1918-19 epidemic, and Strain 36, obtained in February, 1922, may be compared in Figs. 5 and 6. Colonies of these strains on anaerobic blood agar plates are shown in Figs. 8 and 9.

Filterability.—Mention has already been made of Strain 32, which was obtained by direct cultivation of the filtered nasopharyngeal washings from Case 32. Other new strains, obtained from rabbit lung tissues, have passed Berkefeld V candles and initiated growth in culture media.

Pathogenicity.—So far all of the new strains tested (first to eighth generation) have retained their pathogenicity for rabbits. With one strain the process of intratracheal injection, followed by the characteristic infection and recovery of the organism from the lung tissue, has been repeated five times. The three other strains have been recovered in this manner twice. A typical protocol illustrates the clinical and pathological results of intratracheal injection of the strain of *Bacterium pneumosintes* derived from Case 36.

Rabbit A.—Mar. 15, 1922. Leucocytes 12,000, of which 7,680 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a suspension of washed mass culture⁴ of *Bacterium pneumosintes*, the third generation of Strain 36. Mar. 16. Leucocytes 8,400, of which 3,780 were mononuclear cells (Text-fig. 2). Temperature 39.5°C. Mild conjunctivitis. Mar. 17. Leucocytes 6,800, of which 2,244 were mononuclear cells. Temperature 39.8°C. Mild conjunctivitis. Killed. Observable lesions were confined to the lungs and trachea. The lungs were the site of a diffuse hemorrhagic edema and emphysema without any obvious consolidation. Microscopically, the hemorrhages, edema, and emphysema were noted as well as the typical cellular exudation and bronchial lesions similar to those found in the lungs of the rabbit injected with the nasopharyngeal secretions from an influenza patient, and described above (Fig. 7).

In Giemsa-stained preparations of the lung tissue small groups of microorganisms, indistinguishable from *Bacterium pneumosintes*, were found in the ciliary layer

of the bronchial epithelium. Aerobic cultures of the lung tissue showed no growth. Anaerobic cultures in ascitic fluid-rabbit kidney medium yielded pure growths of *Bacterium pneumosintes*.

Locus of Bacterium pneumosintes in Affected Lungs.—We have not heretofore reported direct observations on the presence of *Bacterium pneumosintes* in the lungs of infected rabbits. Frequently in the course of the earlier studies, in impression smears or sections of affected rabbit lungs, stained by Gram's method, with Löffler's methylene blue, or with methylene blue and eosin, minute but questionable bodies had been made out. In view of their inconstancy and the difficulty of demonstrating them with the stains employed, we had not been willing to accord them diagnostic significance. Recently in microscopic sections stained by Giemsa's method, minute blue- or violet-stained bodies, morphologically identical with *Bacterium pneumosintes*, have been found repeatedly in scattered groups in the affected pulmonary tissues. Their usual site is deep in the ciliary margin of bronchial epithelium (Figs. 3 and 4). Less often the bodies are found in mucous and serous exudate between the cilia and in the lumen of the bronchioles, where they are in process of phagocytosis by polymorphonuclear cells and monocytes (Fig. 2). Infrequently they have been found in the interalveolar strands, where they are usually surrounded by an inflammatory mononuclear cell reaction.

These minute and characteristic bodies, which have not been observed in normal rabbit lung tissue, have been clearly made out in various sections of the pulmonary tissues of six rabbits injected with the active agent of the nasopharyngeal secretions of influenza patients. From three of these rabbits pure cultures of *Bacterium pneumosintes* were obtained. The incidence and distribution of these bodies in the lesions caused by *Bacterium pneumosintes*, and their morphological and tinctorial similarity to this microorganism lead us to suppose that they are *Bacterium pneumosintes*.

Reduction of Resistance to Secondary Infection.—A peculiar and significant property of the strains of *Bacterium pneumosintes* obtained in 1918-19 and 1920 was their effect in reducing the resistance of the lung tissues of experimentally infected rabbits to invasion by other bacteria.⁶ Some of the recently isolated strains have been tested in a similar manner and with similar results. For example, after

repeated trials had shown that 3 million pneumococci of an atypical Type II strain, injected intravenously, rapidly disappeared from the blood stream and produced no lesions in rabbits, the tested dose was used in controlled experiments such as the following.

Rabbit A.—May 16, 1922. Leucocytes 6,300, of which 3,090 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a saline solution suspension of *Bacterium pneumosintes*, Strain 34, third generation in mass culture.⁴ May 17. Leucocytes 6,000, of which 1,200 were mononuclear cells. Mild conjunctivitis. Temperature 39.5°C. Injected intravenously with 1 cc. of a saline solution suspension containing 3 million pneumococci, atypical Type II. May 18. Leucocytes 5,600, of which 2,520 were mononuclear cells. Conjunctivitis. Temperature 41.2°C. May 19. Leucocytes 18,800, of which 11,280 were polymorphonuclear cells and 7,520 were mononuclears. Conjunctivitis. Temperature 40.7°C. Blood taken for culture. Killed. The lower right lobe was voluminous, dark, and resistant to the knife. Section revealed a dark and congested cut surface. Fibrinopurulent material exuded from the bronchi. Microscopically, the exudate was found to consist mainly of polymorphonuclears with acidophilic granules and a few mononuclear and respiratory type cells with fibrin and serum. Impression smears of the lung stained by Gram's method showed numerous polymorphonuclear cells containing phagocytosed pneumococci. Free pneumococci seen also. The blood culture and the aerobic and anaerobic cultures of the lungs yielded pure growths of the pneumococcus. Diagnosis: pneumococcus septicemia and pneumonia.

Precisely similar results were obtained with the streptococcus and *Bacillus pfeifferi*. These experiments will be described in detail in the next paper of this series.

Serological Reactions.—The first anti-*pneumosintes* rabbit serum produced was obtained by five weekly injections of a suspension of live organisms (Strain 11) and showed an agglutination titer of 1:160 to 1:320 against the homologous strain.⁷ Other sera, produced with live or killed cultures of Strains 16, 17, and 26, have shown a dilution limit of 1:16 to 1:32 for complete agglutination, even after prolonged immunization. We are inclined to believe that, aside from individual variation in rabbits, the relatively low titer of immune serum to be obtained with *Bacterium pneumosintes* is characteristic of the organism.

For diagnostic purposes this low titer of the anti-*pneumosintes* serum is not objectionable, since the reactions at dilutions of 1:8 and 1:16 are sharp and specific. We have never seen *Bacterium pneu-*

mosintes agglutinate in normal rabbit serum, even undiluted, and the bacterium shows no tendency to spontaneous flocculation in salt solution, remaining in smooth suspension for 24 to 48 hours. Dr. De Kruif, who has done some unreported experiments on the acid agglutination zone of *Bacterium pneumosintes*, informs us that it flocculates out in an unusually narrow zone, lying between pH 3.0 and 3.6, and he infers from this that *Bacterium pneumosintes* is a stable microorganism, not easily agglutinable by immune serum or other chemical or physical agents.

TABLE I.

Agglutination Tests with Old (Strains 17 and 26) and New Strains of Bacterium pneumosintes.

Strain No.	Immune Serum D.				Normal rabbit serum.				Salt solution.
	Dilutions.				Dilutions.				
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	
17	+++	+++	+++	+++	—	—	—	—	—
26	+++	+++	+++	+++	—	—	—	—	—
32	+++	+++	+++	+++	—	—	—	—	—
34	+++	+++	+++	++	—	—	—	—	—
36	+++	+++	+++	+++	—	—	—	—	—
40	+++	+++	+++	+++	—	—	—	—	—

In Table I are given the results of an agglutination test with old and new strains of *Bacterium pneumosintes* against immune rabbit serum produced with one of the old strains. The serum was obtained May 6, 1920, from a rabbit intravenously injected with living cultures of Strain 11. The bacterial suspensions for agglutination were grown in *Bacillus coli* broth,⁸ centrifuged, taken up in salt solution, killed by heat at 56–60°C. for 30 minutes, and standardized by opacity. The tests were set up in successive dilutions in capsules,¹¹ incubated at 55°C. for 1 hour, and stored in the ice box over night before macroscopic examination.

¹¹ Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

As a further test of specificity, two of the old strains derived from Cases 16 and 17¹ and three of the new strains from Cases 32, 34, and 36 were examined for their capacity to absorb specific agglutinins.

Suspensions of the absorbing strains, of ground glass opacity, were distributed into two sets of small test-tubes in lots of 1 cc. These tubes were then centrifuged and the supernatant fluid was carefully pipetted off from the compact sediment. To each of one set of tubes was added 1 cc. of immune serum (made with Strain 16 and obtained Feb. 28, 1922) in a dilution of 1:4, the bacteria were suspended by agitation, and the tubes were incubated for 1 hour at 55°C. After centrifugation, the supernatant serum from the incubated tubes (now absorbed once) was poured on to the sediment of the corresponding culture in the second set of tubes, which were shaken and incubated for the second absorption for 1 hour at 55°C. In this way dilution of the serum was avoided. The serum samples, now twice absorbed, together with an unabsorbed control which had been incubated for 2 hours at 55°C., were then used in the agglutination test shown in Table II.

In the table the columns grouped under A show the agglutination of the various strains in the unabsorbed control serum. In Group B each absorbed serum sample was tested against its own absorbing strain. The tests show that absorption of specific agglutinins had occurred but was not complete. In Group C the serum samples, absorbed with the various strains, were tested against the homologous strain, No. 16. All of the strains had removed specific agglutinins for the homologous strain, but again, absorption was evidently not complete with the amounts of bacteria employed. Finally, in Group D the serum was (incompletely) absorbed with the homologous strain, which removed part of the agglutinins for all of the other strains.

Numerous other agglutination tests with the new strains against serum made with the old ones, and *vice versa*, have proved the antigenic identity of the new strains with the old, and among themselves. If antigenic differences or variations exist by which the strains isolated in 1918-19 and 1920 can be differentiated from those obtained in 1922, we have so far failed to discover them.

Immunological Reactions.—Because the old strains, during long cultivation, had lost their pathogenicity for rabbits, we were able to carry out cross-immunity tests in one direction only; namely, to discover whether injections of the old strains protect against subse-

TABLE II.

Absorption Tests with Old (Strains 16 and 17) and New Strains of Bacterium pneumosintes.

Group A.					Group B.				
Absorbing strains.	Strains agglutinated.	Unabsorbed control serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
Not absorbed.	16	+++	+++	+++	16	16	++	—	—
	17	+++	+++	+++	17	17	+++	+	—
	32	+++	+++	+++	32	32	+	—	—
	34	+++	+++	+++	34	34	+	+	—
	36	+++	+++	+++	36	36	++	+	—

Group C.					Group D.				
Absorbing strains.	Strains agglutinated.	Absorbed serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
17	16	+++	—	—	16	17	+++	++	+
32	16	+++	+	—	16	32	++	+	—
34	16	+++	+	—	16	34	+	—	—
36	16	+++	+	—	16	36	+	—	—

quent infection with the new ones. Two such experiments have been performed and have indicated this protection, as the following protocol shows.

Rabbit A.—Apr. 11, 1922. Injected intratracheally with 3.5 cc. of suspension of centrifuged sediment consisting of live *Bacterium pneumosintes*, Strains 16 and 26, derived from the 1918-19 and the 1920 waves, and grown in *B. coli* broth.⁸ These cultures were at this time not pathogenic for rabbits.⁴ Apr. 24. Leucocytes 12,600, of which 5,544 were mononuclear cells. Temperature 39.7°C. Bled from ear vein for agglutination test. Injected intratracheally with 3 cc. of washed sediment of live mass culture⁴ of *Bacterium pneumosintes*, Strain 34, derived by rabbit passage from Case 34 of the 1922 wave, in the fourth generation. Apr. 25. Leucocytes 12,000, of which 5,760 were mononuclears. Temperature 39.5°C. Conjunctivæ normal. Apr. 26. Leucocytes 10,800, of which 5,724 were mononuclear cells. Temperature 39.5°C. Conjunctivæ normal. Killed. All organs, including lungs, normal. Aerobic and anaerobic cultures of lungs were free from growth.

Rabbit B (Normal Control).—Apr. 24, 1922. Leucocytes 9,000, of which 4,500 were mononuclears. Temperature 39.2°C. Inoculated with the recent living culture of *Bacterium pneumosintes* precisely as in the case of Rabbit A. Apr. 25. Leucocytes 4,800, of which 1,536 were mononuclear cells. Temperature 39.2°C. Conjunctivitis. Apr. 26. Leucocytes 4,600, of which 1,472 were mononuclears. Temperature 39.5°C. Conjunctivitis. Killed. Lungs showed the hemorrhagic edema, emphysema, peculiar cellular exudation, and bronchial lesions characteristic of the action of *Bacterium pneumosintes*. Aerobic cultures of lungs were free from growth; anaerobic culture yielded a pure growth of *Bacterium pneumosintes*.

We have already demonstrated⁶ the presence of agglutinins against *Bacterium pneumosintes* after intratracheal injection of the active influenzal agent with which the rabbit transmission experiments were carried out. In a similar test the serum from Rabbit A, obtained 13 days after the first intratracheal inoculation with the old strains, agglutinated both the old and recent strains of *Bacterium pneumosintes*. The immunity produced by intratracheal injection, even of non-pathogenic strains of *Bacterium pneumosintes*, is therefore demonstrable by serological tests. These observations on protection and antibody formation after intratracheal inoculation agree with those of Besredka,¹² D'Aunoy,¹³ and others.

SUMMARY AND CONCLUSIONS.

From the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza during the recurrence in New York City in January and February, 1922, we have again obtained an active agent, pathogenic for rabbits, and have identified this active agent as *Bacterium pneumosintes*. Four new strains of this micro-organism have been isolated in pure culture and identified with the 1918-19 and 1920 strains on morphological, cultural, and serological grounds. All of the significant characteristics of the old strains, including their effect upon the resistance of the lungs of rabbits to secondary invasion with other bacteria, have been noted in the new strains, which thus have served to confirm and extend our original observations.

¹² Besredka, A., *Ann. Inst. Pasteur*, 1919, xxxiii, 882; 1920, xxxiv, 361.

¹³ D'Aunoy, R., *J. Infect. Dis.*, 1922, xxx, 347.

Addendum.

Reference has already been made in this paper to the discovery of other anaerobic, filter-passing, Gram-negative microorganisms, not *Bacterium pneumosintes*, in nasopharyngeal secretions from human sources. Three of the five cultures which proved not to be *Bacterium pneumosintes* were derived from the filtered nasopharyngeal secretions of supposedly normal persons. What the importance of these microorganisms may be, or whether they have any pathogenic significance we are not prepared to suggest. They indicate, however, that the cultural methods recently employed in these studies may lead to the isolation of a group, or groups, of hitherto undescribed inhabitants of the upper respiratory tract. Our observations may therefore be reported, even though fragmentary, as an indication of interesting opportunities in this field of bacteriology.

Although all the five strains of bacteria, not *Bacterium pneumosintes*, and not pathogenic for rabbits, which have been isolated, are strictly anaerobic, decolorize by Gram's method, and have passed tested Berkefeld V or N filters once or repeatedly, they differ culturally and morphologically among themselves and fall into three apparently unrelated groups.

Group I is represented by a slender vibrio which was obtained from the filtered nasopharyngeal secretions of an undoubted case of epidemic influenza from which *Bacterium pneumosintes* was not obtained.

This vibrio (Fig. 10) is approximately 0.15 to 0.2 micron in thickness, and from 0.5 to 2 microns long, with rounded ends and a smooth curve in contour which may approach a half circle. In the early generations in artificial culture media extremely minute forms were observed, but in later generations the larger forms have predominated. The culture was filtered through a tested Berkefeld V candle in the third generation. This microorganism usually takes the bacterial stain faintly and uniformly throughout. Occasionally polar staining has been observed. In fluid media, such as ascitic-kidney, dextrose broth-kidney, or *Bacillus coli* broth under a vaseline seal, it grows first in minute flakes, which soon settle out as an amorphous or flocculent sediment leaving a clear supernatant fluid. On rabbit blood plates, incubated in an anaerobic jar, it forms pin-head, dis-

crete and confluent colonies, without odor, which do not discolor or precipitate the medium (Fig. 11). The colonies are raised, convex, with an entire edge and a grayish translucency. The organism does not emulsify readily and tends to spontaneous flocculation. Serum from a rabbit immune to this organism does not agglutinate *Bacterium pneumosintes*.

Group II is represented by an extremely minute bacillus (Fig. 12) isolated from a patient with acute follicular tonsillitis. Morphologically this microorganism is not unlike *Bacterium pneumosintes*, except that it shows greater variations in length and thickness. So far we have been unable to obtain constant growths in fluid media, although the strain had survived and possibly multiplied during several weeks incubation in anaerobic blood broth. On anaerobic blood agar plates it forms extremely minute colonies, well shown in Fig. 13. In microscopic tests with the surface growth on blood agar this microorganism has failed to show any agglutination in anti-*pneumosintes* serum. It is not spontaneously agglutinable.

A third group of organisms, differing from the other two, and possibly among themselves, is found in three strains of filter passers obtained from normal throats. Morphologically these strains are also bacillary, with wide variations between the longest and the shortest forms (Fig. 14). They, too, have failed to grow consistently in fluid media, and have been maintained for many generations on anaerobic blood agar plates. The characteristic colonies of these organisms are shown in Fig. 15. A sharp cone is surrounded by a plateau with an irregular edge. These colonies are slightly brownish in color and can thus be distinguished by transmitted light. The representatives of this group flocculate in normal rabbit serum.

These fragmentary observations are reported not because of any supposed relation to *Bacterium pneumosintes*, but as an indication that other microorganisms, which must be differentiated from *Bacterium pneumosintes*, may be encountered under similar conditions. The isolation of these other bacteria has depended upon the use of anaerobic blood agar plates. The failure of all but one of them to grow in fluid media and the fact that they are not pathogenic for rabbits, under the conditions of our experiments, show why they were not encountered during the earlier periods of study, before the blood plates were used.

EXPLANATION OF PLATES.

PLATE 46.

FIG. 1. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with the nasopharyngeal secretions from a patient with early influenza (Case 28). The edema, emphysema, and, in the upper portion, hemorrhage are shown. To be noted also are the distention of blood vessels with blood, the bronchial lesion (lumen filled with serum, leucocytes, and exfoliated necrotic epithelium), and the interalveolar cellular exudate. $\times 250$.

PLATE 47.

FIG. 2. Giemsa-stained section of the lung from a rabbit injected intratracheally with the nasopharyngeal secretions from another influenza patient (Case 30). Lumen of a bronchus. Microorganisms indistinguishable from *Bacterium pneumosintes* are seen at points marked *A*, mixed with precipitate at *B*, phagocytosed by polymorphonuclear cells at *C*, and by an endothelial leucocyte, or monocyte, at *D*. The ciliary margin is indicated by *E*. $\times 1,000$.

FIG. 3. Giemsa-stained section of the lung shown in Fig. 1. A clump of microorganisms indistinguishable from *Bacterium pneumosintes* is seen deep in the ciliary margin. Aerobic cultures of this tissue were free from growth; anaerobic cultures yielded *Bacterium pneumosintes*. $\times 1,000$.

FIG. 4. Giemsa-stained section of the lung from a rabbit intratracheally injected with a mass culture of *Bacterium pneumosintes*, Strain 34, showing microorganisms in the ciliary layer of a bronchus. $\times 1,000$.

FIG. 5. Stained preparation of *Bacterium pneumosintes*, Strain 16, from the 1918-19 epidemic, in fresh tissue dialysate medium.⁹ Cultivated artificially for 2½ years. To be compared with Fig. 6. $\times 1,000$.

FIG. 6. Stained preparation of *Bacterium pneumosintes*, Strain 36, from the 1922 wave, in fresh tissue dialysate medium. Cultivated artificially for 2 months. To be compared with Fig. 5. $\times 1,000$.

PLATE 48.

FIG. 7. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with a culture of *Bacterium pneumosintes*, Strain 36. To be noted are the lesions similar to those shown in Fig. 1. $\times 250$.

PLATE 49.

FIG. 8. Colonies of *Bacterium pneumosintes*, Strain 16, from the 1918-19 wave, grown anaerobically on the surface of blood agar. $\times 10$.

FIG. 9. To be compared with Fig. 8. Similar colonies of *Bacterium pneumosintes*, Strain 36, from the 1922 wave. $\times 10$.

FIG. 10. Showing the morphology, after repeated subplants, of anaerobic, filter-passing, Gram-negative bacteria of Group I. From a culture in ascitic fluid-fresh tissue medium. $\times 1,000$.

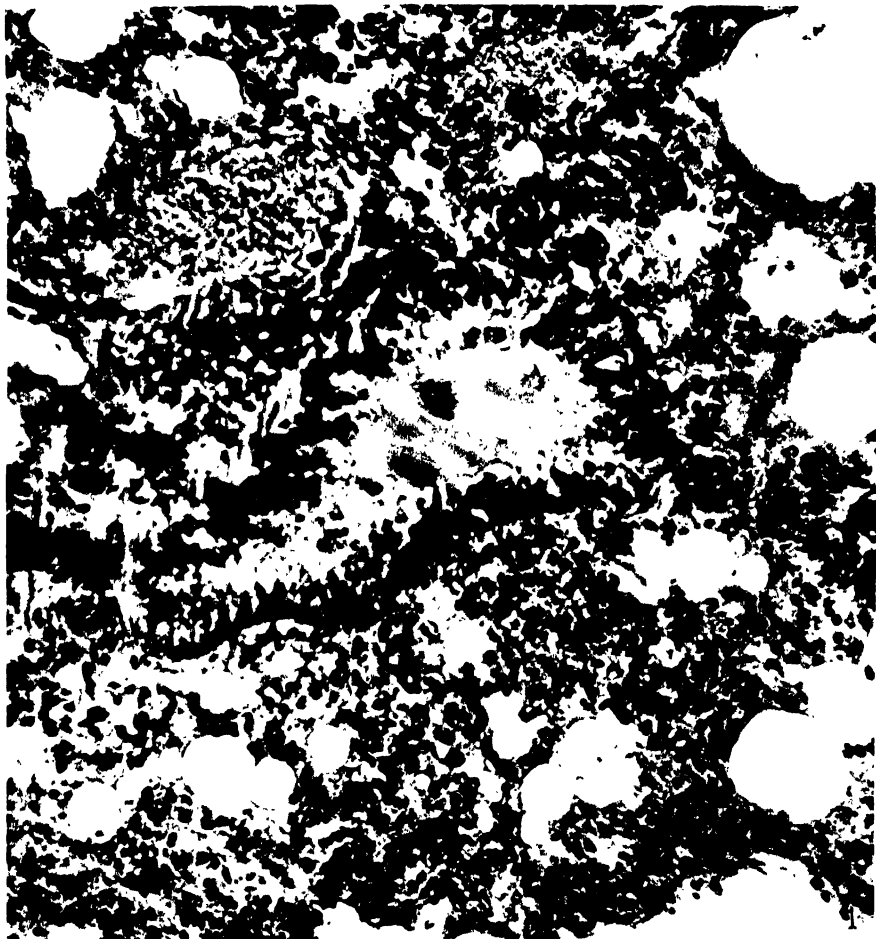
FIG. 11. Colonies of the culture shown in Fig. 10, grown anaerobically on the surface of blood agar. $\times 10$.

FIG. 12. Morphology of anaerobic, filter-passing, Gram-negative bacteria of Group II. $\times 1,000$.

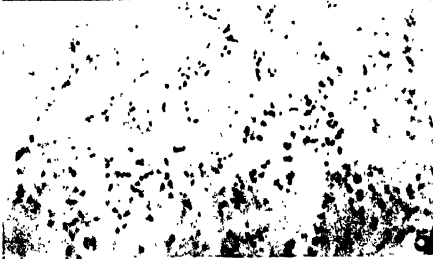
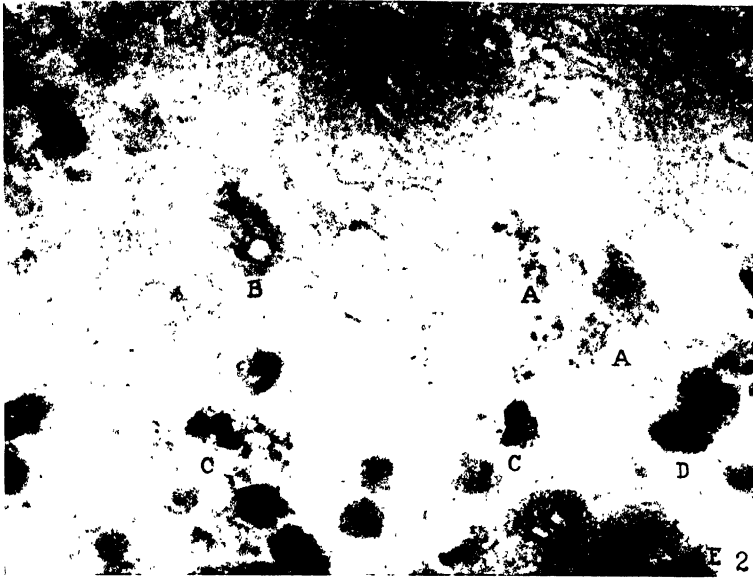
FIG. 13. Colonies of the culture shown in Fig. 12, grown anaerobically on the surface of blood agar. $\times 10$.

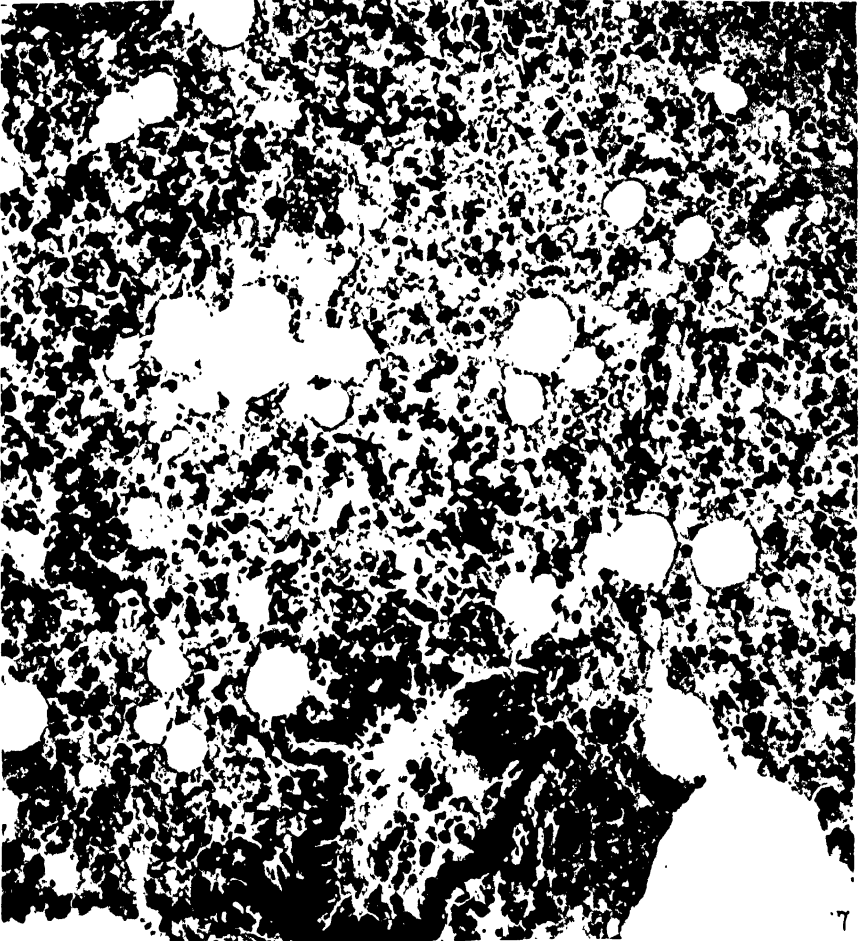
FIG. 14. Anaerobic, filter-passing, Gram-negative bacteria of Group III. $\times 1,000$.

FIG. 15. Colonies of the culture shown in Fig. 14, grown anaerobically on the surface of blood agar. $\times 10$.

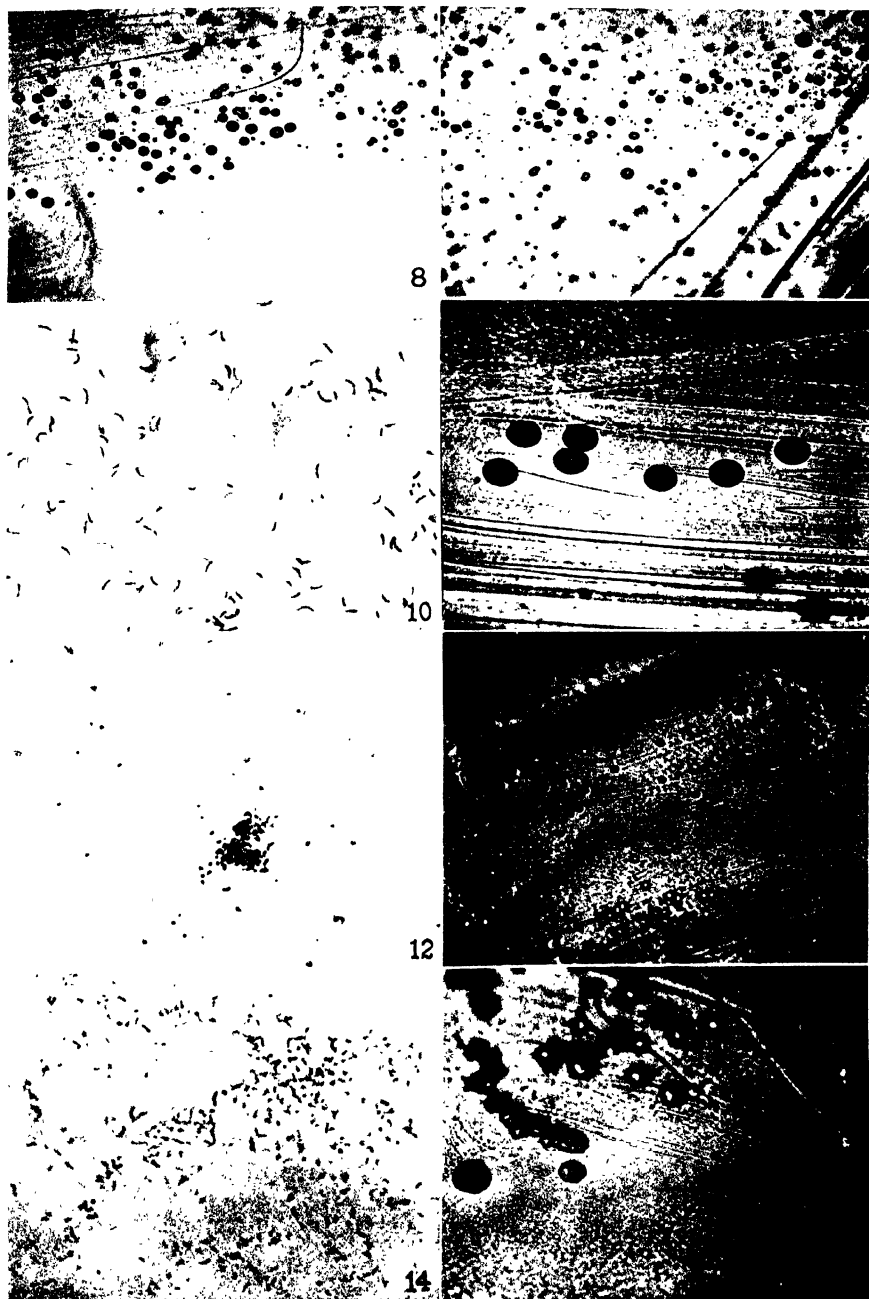


(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)





(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)



(Olitsky and Gates: Nasopharyngeal secretions from influenza IX.)

EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

X. THE IMMUNIZING EFFECTS IN RABBITS OF SUBCUTANEOUS INJECTIONS OF KILLED CULTURES OF *BACTERIUM PNEUMOSINTES*.

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(Received for publication, July 18, 1922.)

It is evident from the results of the serological experiments described in Papers VII¹ and IX² of these studies that the reaction of the animal body to intratracheal and intravenous injections of living *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients involves the production of serum antibodies. Evidence of the protective effects of such injections against subsequent infection with *Bacterium pneumosintes* has also been reported.^{2,3}

It appears, therefore, that *Bacterium pneumosintes* has the antigenic character common to bacteria in general, and that the mechanism of protection against it is similar to that which comes into play in the case of infections with other pathogenic microorganisms. If this is so, it follows that subcutaneous injections of killed cultures of *Bacterium pneumosintes* may be expected to induce a similar protective reaction. Recently, we have had the opportunity to put this hypothesis to experimental test. A study of the serological and immunological reactions of rabbits to prophylactic subcutaneous injections of killed *Bacterium pneumosintes* is reported in the present paper.

The opportunity for such a study was afforded by the recurrence of epidemic influenza in New York City in the early months of 1922. This recurrence provided us with four new strains of *Bacterium*

¹ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.

² Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxvi, 501.

³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.

pneumosintes which had not undergone prolonged artificial cultivation and were, therefore, pathogenic for rabbits.² With these the protective effects of subcutaneous injections could be tested, both against subsequent infection of the lungs with *Bacterium pneumosintes*, and, as a corollary, against the secondary invasion of the lungs by other bacteria. The study also furnished evidence of the local and general reactions which might be expected to follow the subcutaneous injection of killed cultures of *Bacterium pneumosintes*, and thus prepared the way for similar observations in man.

Methods.

Preparation of the Killed Cultures of Bacterium pneumosintes.—The experimental evidence^{1,2,3} points to the antigenic identity of all of our strains of *B. pneumosintes*, whether derived from the epidemic of 1918–19, the recurrence of 1920, or the recent recurrence of 1922. It did not seem advisable, therefore, to prepare a polyvalent vaccine of all the available strains, but, in order to include possible antigenic differences, which the serological tests did not disclose, two cultures were chosen and used in equal quantities,—Strain 17, isolated in 1918 directly from the nasopharyngeal secretions of an influenza patient, and Strain 34, obtained in February, 1922, from lung tissue derived from a rabbit in the second passage of the active agent from Case 34.

The organisms were grown for 3 to 5 days in 100 cc. Florence flasks of *B. coli* broth,² seeded with 1 cc. of a dextrose broth-rabbit kidney culture of Strain 17 or 34. After centrifugation the supernatant medium was decanted and the sediment suspended in 0.85 per cent salt solution and heated for 30 minutes at 56–60°C. The suspension was then standardized on an opacity scale and tested for sterility.

Standardization of the Suspensions.—Because of wide differences in the size of bacteria, the standardization of suspensions by counting methods does not afford comparable data on the amount of bacterial substance present, especially since the accepted error in counting methods is often as great as 25 per cent. On the other hand, it is convenient to speak of bacterial suspensions in terms of the bacterial count. What is lacking is a comparative standard to which bacteria of various sizes can be referred.

We have adopted *Staphylococcus albus* as a convenient standard and have correlated the bacterial count and the opacity of its suspensions in a number of tests. Using the Wright method for counting and the depth of disappearance readings described by one of us (Gates⁴), it was found that 1,000 million heat-killed staphylococci corresponded to a corrected opacity of 3.47 cm.

⁴ Gates, F. L., *J. Exp. Med.*, 1920, **xxi**, 105.

Regardless of the actual number of *B. pneumosintes* in a given suspension, we have standardized the killed cultures on the staphylococcus scale. For example, a suspension of *B. pneumosintes* which has a corrected opacity of 3.47 cm. is said to contain $\frac{1,000 \text{ million}}{\text{S. E.}}$ organisms (Staphylococcus Equivalent). In other words, the amount of bacterial substance present is assumed to correspond approximately to the amount contained in a suspension of 1,000 million staphylococci per cc. Suspensions of *B. pneumosintes*, standardized by this method, have been checked by comparison with equivalent quantities of *Staphylococcus albus*, *B. typhosus*, and Meningococcus Type A in Hopkins tubes.⁵ The amounts of bacterial sediment obtained corresponded within the limit of observation of the tubes.

Procedure.—Twenty-six apparently healthy adult rabbits were given three subcutaneous injections of heat-killed *Bacterium pneumosintes*, at intervals of 5 days. With the Staphylococcus Equivalent as a measure, the doses chosen were $\frac{2,000 \text{ million}}{\text{S. E.}}$, $\frac{4,000 \text{ million}}{\text{S. E.}}$, and $\frac{4,000 \text{ million}}{\text{S. E.}}$ *Bacterium pneumosintes*, a total for each animal of $\frac{10,000 \text{ million}}{\text{S. E.}}$. The injected suspensions contained equal quantities of Strains 17 and 34, in 1 to 2 cc. of physiological saline solution. In order to localize the subcutaneous reaction, the injections were made through a shaved area, cleansed with soap and water and alcohol, on the most dependent portion of the abdomen.

Reactions.—None of the rabbits showed any significant local reactions or rise in temperature following the first injection of $\frac{2,000 \text{ million}}{\text{S. E.}}$ organisms. After the second injection $\left(\frac{4,000 \text{ million}}{\text{S. E.}}\right)$ most of the animals showed a mild, local reaction, consisting of redness, heat, and swelling which persisted for 2 days. The third injection rarely caused local symptoms. No general symptoms referable to the inoculations were noted. One rabbit was found dead on the 2nd day after the third injection, and two others were found to be suffering from an infection prevalent among the stock rabbits, and were killed.

⁵ Hopkins, J. G., *J. Am. Med. Assn.*, 1913, lx, 1615.

Four other rabbits were given the total dose of $\frac{10,000 \text{ million}}{\text{S. E.}}$

killed culture of *Bacterium pneumosintes* in a single subcutaneous injection. Two of these rabbits developed localized areas of heat, redness, and swelling. The other two showed practically no local reaction. Blood counts made on these animals before and after injection showed no immediate change in the total leucocyte count, or in the proportion of mononuclear cells, such as is characteristic of infection with the living organisms. The body temperatures were not affected by the injection.

Agglutinin Formation.

From 10 to 32 days after the third subcutaneous injection, nineteen of the rabbits were used in the protection experiments to be described. The blood serum of fifteen of them was tested for the presence of specific agglutinins, 10 to 27 days after the final injection. Of these sera, four failed to agglutinate the homologous strains of *Bacterium pneumosintes* in the lowest dilution tested, 1:4. Five sera, in this dilution, showed agglutinins against one or both strains. Five other sera agglutinated the strains when diluted 1:8, and one in a dilution of 1:16. In view of the individual variations in response among rabbits, and the relatively low agglutinogenic properties of *Bacterium pneumosintes*² these results are what would be expected. They indicate that the subcutaneous injection of killed cultures of *Bacterium pneumosintes* induces antibody formation. But the critical test of immunization is not the titer of serologically demonstrable antibodies, but protection against subsequent infection.

As a preliminary to the series of protection experiments with the subcutaneously inoculated animals, the four rabbits that were injected with a single dose of $\frac{10,000 \text{ million}}{\text{S. E.}}$ organisms were tested for

lowered resistance on the day following inoculation. Attention has repeatedly been called to one of the characteristic features of infection with *Bacterium pneumosintes*; namely, the loss of resistance to secondary infection which occurs in the injured lung.^{6,7} In

⁶ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.

⁷ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.

view of the nature of the lesions caused by *Bacterium pneumosintes* in the pulmonary tissues, and the localization in these damaged tissues of other bacteria, injected intravenously, it is natural to suppose that it is the local disorganization and destruction of the lung structure that opens the way to secondary infections. Nevertheless, it was conceivable that *Bacterium pneumosintes* might have some inherent, specific property of promoting infections by other organisms and it seemed important to know whether, in the absence of a pulmonary lesion, the presence of *pneumosintes* protein in the body might reduce the natural resistance to other bacteria during what is commonly called a negative phase.

To test this possibility, two of the four rabbits that received 10,000 million killed organisms in one dose were injected intravenously

S. E.

on the following day with a dose of atypical Type II pneumococcus which was harmless to normal animals but infective for those suffering from a pulmonary lesion induced by *Bacterium pneumosintes*.

One of the animals remained well, gave negative blood cultures on the 2nd day, and, when killed for autopsy on that day, showed no lesions of the lungs, cultures of which remained sterile. The other rabbit died on the day following injection. No obvious lesions were found. The blood culture, post mortem, yielded the bacillus of rabbit septicemia. This infection, from which two other vaccinated rabbits were found to be suffering, was prevalent among the stock. Since the subcutaneous injection of killed cultures of *Bacterium pneumosintes* apparently did not lower the resistance of the two animals to the pneumococcus, these accidental infections presumably bear no relation to the problem in hand.

The third rabbit was similarly injected with a non-infective dose of *Streptococcus haemolyticus*, and the fourth with *Bacillus pfeifferi*. Neither streptococcus nor Pfeiffer's bacillus could subsequently be recovered by blood culture, and at autopsy the organs of the rabbits, including the lungs, appeared normal. Cultures of the lung tissue were negative.

Protection Experiments.

Experiments to test the protective efficacy of the subcutaneous injections of killed cultures were divided into three groups.

The vaccinated rabbits in Group A were injected intratracheally with the active agent of rabbit passages in the 1922 series, in doses that proved infective for normal control animals. The vaccinated rabbits in Group B were intratracheally injected with mass cultures of pathogenic (1922) strains of *Bacterium pneumosintes*. The vaccinated rabbits in Group C were injected intratracheally with *Bacterium pneumosintes*, and then intravenously with other bacteria, in doses which, following a similar intratracheal injection of *Bacterium pneumosintes*, caused pneumonia in uninoculated controls. Typical protocols may be cited to illustrate each group.

Group A. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of the Active Agent of Rabbit Passages. Experiment 1.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 9. Leucocytes 5,000, of which 2,200 were mononuclear cells. Temperature 39.3°C. May 10. Injected intratracheally with 3 cc. of a salt solution suspension of ground lung tissue derived from a rabbit in the third passage of the active agent from a recent case of early epidemic influenza (Case 28³). May 11. Leucocytes 5,200, mononuclears 3,224. Temperature 39.3°C. May 12. Leucocytes 9,000, mononuclears 5,670. Killed. Lungs and other organs show no lesions. Aerobic and anaerobic cultures of lung tissue yielded no growth.

Rabbit B (normal control): May 9. Leucocytes 14,400, of which 6,336 were mononuclear cells. Temperature 39.3°C. Conjunctivæ normal. May 10. Intratracheally injected with 3 cc. of the ground lung suspension given to Rabbit A. May 11. Leucocytes 11,000, mononuclears 4,840. Temperature 39.4°C. Conjunctivitis. May 12. Leucocytes 8,400, mononuclears 2,856. Temperature 39.5°C. Conjunctivitis. Killed. Only the lungs showed lesions. Both lungs were the site of a hemorrhagic edema and an emphysema, without consolidation. Microscopically the affected tissues showed marked edema of the interalveolar strands with a cellular exudate of mononuclear and polymorphonuclear cells. The lumina of the bronchi were filled with serum, leucocytes, and cells from the necrotic and exfoliated epithelium. Among the cilia of the bronchial epithelium clumps of minute bacteria, morphologically similar to *B. pneumosintes*, were noted. Anaerobic, as well as aerobic cultures, failed to yield a growth, however. A repetition of this experiment gave the same results.

The experiments illustrated by these typical instances show that subcutaneous injections of killed cultures of *Bacterium pneumosintes* cause the development of an effective resistance to pathogenic doses of the active agent derived from the nasopharyngeal secretions of influenza patients. The identity of this active agent with *Bacterium*

pneumosintes has already been demonstrated. Tissue suspensions were used because it was thought that *Bacterium pneumosintes* contained in the lungs of affected rabbits after repeated passages might be enhanced in virulence and pathogenicity, and hence afford a more severe test of resistance than was to be obtained with the laboratory strains which were studied in Group B.

Group B. To Test the Resistance of Vaccinated Rabbits to Pathogenic Doses of . pneumosintes. Experiment 2.—Rabbit A (vaccinated): Last subcutaneous injection Apr. 29, 1922. May 15. Leucocytes 7,200, of which 2,880 were mononuclear cells. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a salt solution suspension of *B. pneumosintes*, Strain 34, representing the third generation in mass culture. May 16. Leucocytes 8,000, mononuclears 4,160. Temperature 39.2°C. Conjunctivæ normal. May 17. Leucocytes 7,200, mononuclears 4,176. No change in temperature. Conjunctivæ normal. Killed. No lesions found in the lungs or other organs. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 15. Leucocytes 12,000, of which 5,760 were mononuclear cells. Temperature 38.5°C. Conjunctivæ normal. Injected precisely as was Rabbit A. May 16. Leucocytes 10,000, mononuclears 4,400. Temperature 39.5°C. Conjunctivitis. May 17. Leucocytes 6,000, mononuclears 2,700. Temperature 39.6°C. Killed. The lungs showed the hemorrhagic edema, emphysema, cellular reaction, and bronchial lesions already described as characteristic of the effects of *B. pneumosintes*.⁸ Among the cilia of the bronchial epithelium and in the interalveolar tissues, numerous clumps of minute microorganisms, morphologically identical with *B. pneumosintes*, were found. Aerobic cultures of the lung tissue showed no growth; anaerobic cultures yielded *B. pneumosintes*.

This experiment is typical of the five in Group B. In spite of the fact that a large dose of a pathogenic strain of *Bacterium pneumosintes* was employed, the protection afforded by the previous subcutaneous injections of killed culture was sufficient to prevent invasion of the lung.

Since the intratracheal injections of *Bacterium pneumosintes* failed to infect and to produce pathological lesions in the lungs of the vaccinated rabbits in Groups A and B, these animals presumably would have shown a normal resistance to pulmonary infection with other bacteria. The rôle of secondary infections in human influenza is so

⁸ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

important, however, and the reduction of pulmonary resistance induced by *Bacterium pneumosintes* in experimental animals is so characteristic and significant, that we were led to test the resistance of vaccinated rabbits to other bacteria, following intratracheal injections of *pneumosintes* cultures. These experiments comprise Group C of the present series and were carried out with an atypical Type II pneumococcus, a hemolytic streptococcus, and *Bacillus pfeifferi*.

In this group of experiments the procedure described in Paper V of this series⁷ was employed. First, cultures of atypical Type II pneumococcus, *Streptococcus hæmolyticus*, and *Bacillus pfeifferi* were injected intravenously into normal rabbits and a non-infective dose was determined in each case. Then the pathogenicity of mass cultures of the 1922 strains of *Bacterium pneumosintes*, injected intratracheally into normal rabbits, was confirmed (Control Rabbit B, Group B). Finally the animals previously prepared by vaccination, and normal controls, were injected intratracheally with the tested mass cultures of *Bacterium pneumosintes*, and on the following day each was injected intravenously with the dose of one of the organisms above mentioned that had proved non-infective for normal rabbits. The results of these experiments are illustrated by the following protocols.

Group C. To Test the Resistance of Vaccinated Rabbits to Infection with Other Bacteria Following the Intratracheal Injection of B. pneumosintes. Experiment 3. B. pneumosintes and the Pneumococcus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 16. Leucocytes 9,300, of which 2,140 were mononuclears. Temperature 39.8°C. Injected intratracheally with 3 cc. of a mass culture of *B. pneumosintes* similar to that used in Experiment 2. May 17. Leucocytes 11,400, mononuclears 4,450. Temperature 39.9°C. Injected intravenously with 3 million atypical Type II pneumococci, suspended in 1 cc. of saline solution. (This was the tested non-infective dose for this culture of pneumococcus.) May 18. Leucocytes 7,600, mononuclears 3,570. Temperature 40.1°C. May 19. Leucocytes 12,000, mononuclears 5,760. Temperature 40.2°C. May 20. Leucocytes 8,600, mononuclears 4,730. Temperature 40°C. Blood taken for culture yielded no growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue remained sterile.

Rabbit B (normal control): May 16. Leucocytes 6,300, of which 3,090 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, as was Rabbit A. May 17. Leucocytes 6,000, mononuclears 1,200. Temperature 39.5°C. Conjunctivitis. Injected intra-

venously with 3 million pneumococci, as was Rabbit A. May 18. Leucocytes 5,600, mononuclears 2,520. Temperature 41.2°C. Conjunctivitis. May 19. Leucocytes 18,800, mononuclears 7,520, polymorphonuclears 11,280. Temperature 40.7°C. Conjunctivitis. Blood culture made on this day yielded the pneumococcus. Killed. Lungs voluminous with edema and emphysema and showed numerous hemorrhages. Lower right lobe dark, solid, and resistant to section. Fibropurulent discharge from bronchi. Microscopically, hemorrhage, edema, and emphysema were noted in some areas. Other areas were the site of a peribronchial consolidation and polymorphonuclear exudate. Impression smears of the cut lung showed numerous polymorphonuclear cells, and free and phagocytosed pneumococci. Aerobic and anaerobic lung cultures yielded pneumococci. Diagnosis: pneumococcus septicemia and beginning pneumonic consolidation.

This experiment is one of three in which it was found that the pneumococcus, injected intravenously, following an intratracheal injection of *Bacterium pneumosintes*, produced a septicemia and pneumococcus pneumonia in control rabbits but not in rabbits previously vaccinated with killed cultures of the primary organism. One experiment was complicated by the presence of *Bacillus leptosepticus* in the lung tissues, but not in the blood of the vaccinated animal. The lungs of this rabbit showed no pneumonic consolidation, and cultures of lung and blood were negative for the pneumococcus.

Experiment 4. B. pneumosintes and Streptococcus hemolyticus.—Rabbit A (vaccinated): Last injection May 2, 1922. May 22. Leucocytes 5,800, of which 3,070 were mononuclears. Temperature 39.7°C. Conjunctivæ normal. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*, similar to that used in Experiments 2 and 3. May 23. Leucocytes 6,800, mononuclears 3,400. Temperature 39.3°C. Conjunctivæ normal. Injected intravenously with a saline solution suspension of *Streptococcus hemolyticus* containing 1 million microorganisms (the tested non-infective dose for this culture). May 24. Leucocytes 8,400, mononuclears 5,376. Temperature 39.2°C. Conjunctivæ normal. May 25. Leucocytes 6,600, mononuclears 3,498. Temperature 39.2°C. Conjunctivæ normal. Blood cultures taken on this day remained sterile. May 26. Leucocytes 6,600, mononuclears 3,894. Temperature 39.4°C. Conjunctivæ normal. Blood culture free from growth. Killed. Lungs and other organs appeared normal. Aerobic and anaerobic cultures of the lung tissue yielded no growth.

Rabbit B (normal control): May 22. Leucocytes 7,600, of which 2,812 were mononuclears. Temperature 38.4°C. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. May 23. Leucocytes 7,000, mononuclears 1,680. Temperature 39.5°C. Conjunctivitis. Injected intravenously with

1 million *Streptococcus hæmolyticus*. May 24. Leucocytes 8,000, mononuclears 2,240. Temperature 38.9°C. Conjunctivitis. May 25. Leucocytes 5,400, mononuclears 3,240. Temperature 39°C. Conjunctivæ normal. Blood culture positive for *Streptococcus hæmolyticus*. May 26. Leucocytes 6,800, mononuclears 1,700. Temperature 39.4°C. Conjunctivæ normal. Blood culture yielded *Streptococcus hæmolyticus*. Killed. Lungs showed diffuse consolidation of contiguous parts of left upper and lower lobes (red hepatization). Remainder of lung voluminous, edematous, and emphysematous, and showed a number of small hemorrhages on surface and on section. An impression smear showed numerous polymorphonuclear cells and a few streptococci. Cultures of the lung yielded *Streptococcus hæmolyticus*. Diagnosis: streptococcus septicæmia and pneumonia.

Two other similar experiments with *Bacterium pneumosintes* and *Streptococcus hæmolyticus* gave similar results. As in the experiments with the pneumococcus, the previous vaccination of the experimental rabbits with killed cultures of *Bacterium pneumosintes* protected the animals against pathogenic doses of the living organisms; and in the absence of a pulmonary lesion, due to *Bacterium pneumosintes*, they maintained their normal resistance to secondary infection with the streptococcus.

Experiment 5. B. pneumosintes and B. Pfeifferi.—Rabbit A (vaccinated): Last injection May 27, 1922. June 6. Leucocytes 9,000, of which 4,320 were mononuclears. Temperature 39.7°C. Injected intratracheally with 3 cc. of a saline solution suspension of a mass culture of *B. pneumosintes*. June 7. Leucocytes 11,600, mononuclears 4,872. Temperature 39.5°C. Conjunctivæ normal. Injected intravenously with 7.5 billion *B. Pfeifferi* (the tested non-infective dose of this culture) in 1 cc. of saline solution. June 8. Leucocytes 12,800, mononuclears 6,400. Temperature 39.5°C. Conjunctivæ normal. Blood culture remained sterile. June 9. Leucocytes 9,000, mononuclears 5,040. Temperature unchanged. Conjunctivæ normal. Blood cultures yielded no growth. Killed. The lungs and other organs appeared normal. Aerobic and anaerobic cultures showed no growth.

Rabbit B (normal control): June 6. Leucocytes 14,000, of which 7,420 were mononuclears. Temperature 39.2°C. Conjunctivæ normal. Injected intratracheally with *B. pneumosintes*, precisely as was Rabbit A. June 7. Leucocytes 12,800, mononuclears 3,200. Temperature 39.6°C. Conjunctivitis. Injected intravenously with 7.5 billion *B. Pfeifferi*. June 8. Leucocytes 44,000, of which 5,300 were mononuclears and 38,700 were polymorphonuclear cells. Temperature 39.9°C. Conjunctivitis. Blood culture yielded *B. Pfeifferi* in pure culture. June 9. Leucocytes 22,800, mononuclears 4,788, polymorphonuclears 18,012. Temperature 39.9°C. Conjunctivitis. Blood culture positive for *B. Pfeifferi*.

Killed. Right lower lobe of lungs dark, congested, resistant to section, with a purulent exudate in the bronchi. Histological examination revealed edema, hemorrhages, emphysema, and peribronchial and lobular consolidation. The so-called areas contained many polymorphonuclear cells, with some mononuclears and respiratory epithelium in a matrix of serum and fibrin. In some areas the bronchi were filled with leucocytes, serum, and fibrin, surrounded by a necrotic and exfoliated epithelium. Impression smears showed many polymorphonuclear cells and intra- and extracellular Gram-negative bacilli, resembling *B. Pfeifferi*. Anaerobic cultures of the lung were negative, although minute organisms, resembling *B. pneumosintes*, were seen in the ciliary epithelium of affected bronchi. Diagnosis: septicemia and pneumonia, due to *B. Pfeifferi*.

Six similar experiments were made in this series. The results of four of these corresponded to those illustrated above. In the other two experiments the subcutaneous injections failed to protect the animals, and the intratracheal injection of live *Bacterium pneumosintes*, followed by the intravenous injection of *Bacillus Pfeifferi* induced a pulmonary infection to which the rabbits succumbed.

The failure of the vaccine to protect in these two instances may be attributed to individual susceptibility on the part of the rabbits, or possibly to the rather arbitrary dose of *Bacterium pneumosintes* employed. In each experiment the suspension of *Bacterium pneumosintes* for intratracheal injection was divided between the vaccinated and the control animals, 3 cc. to each, but in experiments with different cultures the potentialities of the dose employed may well have varied. Since the control animals in every instance were infected, it is certain that at least one infective dose was given in all, but in some experiments the rabbits may have received several multiples of this minimum. Thus the experiments may have been much more severe tests of the protection afforded by subcutaneous vaccination than the protocols indicate, and it was to be expected that in some instances protection would be incomplete.

SUMMARY AND CONCLUSIONS.

A series of rabbits was subcutaneously injected with three measured doses of killed cultures of two strains of *Bacterium pneumosintes* derived from the nasopharyngeal secretions of influenza patients. These rabbits were subsequently tested for the development of serum

antibodies and for the presence of an induced immunity to the living organisms, with the following results.

The serum of eleven of fifteen rabbits, tested from 10 to 27 days after the final subcutaneous injection, specifically agglutinated *Bacterium pneumosintes*, whereas normal rabbit serum did not.

Nineteen vaccinated rabbits were subjected to protection experiments. Two of them were unaffected by an intratracheal injection of *Bacterium pneumosintes*, contained in the lung tissues of previously infected animals, in a dose which typically affected the control rabbits. Fifteen of the other seventeen proved to be completely resistant when tested by intratracheal injections of *Bacterium pneumosintes* cultures that produced typical infections in the controls. Ten of these fifteen rabbits were injected intravenously with living cultures of pneumococcus, *Streptococcus hæmolyticus*, or *Bacillus pfeifferi* in doses which were non-infective under normal conditions, but infective, as experience has shown, in the presence of a primary lesion caused by *Bacterium pneumosintes*. In none of these animals did infection develop. The two remaining rabbits of the seventeen were not protected against *Bacterium pneumosintes* by the vaccination, and they further developed a secondary pulmonary infection with *Bacillus pfeifferi* after its intravenous injection. Control rabbits similarly injected intratracheally with *Bacterium pneumosintes*, and then intravenously with the pneumococcus, streptococcus, or *Bacillus pfeifferi* in doses that had proved non-infective for normal rabbits, uniformly developed a secondary infection with these organisms.

The mildness of the local reactions and the absence of general signs, following vaccination with *Bacterium pneumosintes*, indicate that similar injections would be well tolerated in man. There is no evidence that the subcutaneous injection of large doses of the heat-killed organisms reduces the resistance of the animal body to infections with other bacteria. In single rabbit experiments the resistance to intravenously injected pneumococci, streptococci, or *Bacillus pfeifferi* has been found unreduced immediately after vaccination with *Bacterium pneumosintes*.

DIFFERENCES BETWEEN MITOCHONDRIA AND BACTERIA.

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(Received for publication, June 10, 1922.)

The suggestion that the granular and thread-like bodies, which are called mitochondria and are known to occur in almost all active cells from protozoa to man and from fungi to angiosperms, are in reality symbiotic microorganisms, would, if verified, exercise a profound influence in biology and medicine. Yet it has been repeatedly advanced in the literature.

First in 1890, Altmann¹ published a detailed account of his researches made with the help of a new method of fixation in a mixture of equal parts of 5 per cent potassium bichromate and 2 per cent osmic acid, staining in aniline fuchsin and differentiating in picric acid. By this technique the existence of mitochondria (and other granulations) was revealed in many different kinds of cells. To designate them he introduced the term bioblasts, indicating his belief that they are elementary microorganisms responsible for vital phenomena, embedded in a lifeless ground substance; but since he presented no more convincing evidence than a certain resemblance in form between the granules and bacteria, his somewhat involved theoretical considerations were not well received. They came, moreover, at a time when attention was directed toward the nucleus through studies in heredity, and the tremendous impetus of Fischer's² studies on the synthesis of the protein molecule lent encouragement to the view that vital phenomena might soon receive their explanation in terms of physics and chemistry. It is not surprising, therefore, that Altmann's really valuable objective findings were forgotten and investigations along this line received a decided setback.

Again in 1918 Portier³ published detailed investigations purporting to show that mitochondria are symbiotic microorganisms, for which he proposed the term *symbiotes*. His contention was based upon a very careful review of the

¹ Altmann, R., *Die Elementarorganismen und ihre Beziehungen zu den Zellen*, Leipzig, 1890.

² Fischer, A., *Fixierung, Färbung und Bau des Protoplasmas*, Jena, 1899.

³ Portier, P., *Les symbiotes*, Paris, 1918.

literature, but his comparison between mitochondria and bacteria is lacking in directness. For details on the mitochondrial side he has relied upon information given by others. He has not compared the morphology and staining reactions of mitochondria and bacteria occurring side by side within the same piece of tissue. His view has been supported by his colleague Bierry⁴ and very severely criticized on general grounds by Regaud,⁵ Guilliermond,⁶ and Laguesse,⁷ who, however, did not subject the problem to the test of a direct comparison.

More recently Wallin,⁸ in 1922, has published observations upon the staining of bacteria with mitochondrial methods and upon the reactions of bacteria to chemical treatment which have led him independently to advance an hypothesis, like that of Portier, according to which all mitochondria are symbiotic bacteria. His is the first attempt to make a detailed microchemical study, but, like Portier,³ the comparison which he institutes is indirect, for, obviously, the application of mitochondrial methods to bacteria means but little unless they are at the same time applied to mitochondria. From his account as it stands, it would be possible to make out a fairly strong case against the validity of his contention. For example, he notes the fact that alcohol is given as a solvent for mitochondria;⁹ he also records the observation that human tubercle bacilli are not destroyed by immersion for 5 hours in 95 per cent alcohol;¹⁰ yet he emphasizes a similarity in reaction between mitochondria and bacteria.¹¹ The variable resistance of bacteria to the ingredients of mitochondrial fixatives, which he has used separately and in such dilution as to produce extensive ionization, indicates further dissimilarity. One would hardly expect any traces of mitochondria to remain (as he finds the bacteria do) after treatment for 6 hours with 0.5 per cent acetic acid, with 1 per cent formalin, or with 0.5 per cent potassium bichromate.

Although the evidence presented by Altmann,¹ Portier,³ and Wallin⁸ is not convincing, certain inquiries which we have received indicate that the discussion provoked may easily lead investigators from the main problem, which is, we take it, the elaboration of new methods for the determination of the chemical constitution of mitochondria as a prerequisite to the study of their rôle in cellular physiology and pathology. Particularly is this true since the problem lies, as it were, between the sciences of cytology and bacteriology, so that but few investigators can be familiar with both sides of the question. In physiology the cell has come to be considered as a vital unit in which it is not profitable to attempt to distin-

⁴ Bierry, H., *Compt. rend. Soc. biol.*, 1919, lxxxii, 131, 312.

⁵ Regaud, C., *Compt. rend. Soc. biol.*, 1919, lxxxii, 244.

⁶ Guilliermond, A., *Compt. rend. Soc. biol.*, 1919, lxxxii, 309.

⁷ Laguesse, E., *Compt. rend. Soc. biol.*, 1919, lxxxii, 337.

⁸ Wallin, I. E., *Am. J. Anat.*, 1922, xxx, 203.

⁹ Wallin,⁸ p. 204.

¹⁰ Wallin,⁸ p. 213.

¹¹ Wallin,⁸ p. 221.

guish between living and non-living constituents, as Hopkins¹² clearly stated in 1913. To admit, in terms of the evidence presented, that practically all cells contain elements endowed with independent vitality would force a complete readjustment in our ideas of the organization of living matter and in the application to it of the laws of physics and chemistry. In pathology these views, if verified, would have a distinct bearing upon our conception of the significance of *Rickettsia* bodies in typhus fever, trench fever, and other conditions and would leave us in some doubt regarding the criteria to be employed in the identification of microorganisms the nature of which remains obscure.

Thus far the only reaction claimed to be specific for mitochondria is that of coloration with Janus green B (diethylsafranineazodimethylaniline chloride). Wallin⁸ obtained a coloration of bacteria when this stain was employed in a concentration of 1:10,000 in physiological saline solution and uses this observation as a basis for his contention of a similarity between mitochondria and bacteria. But the comparison which he has made is not direct because he apparently relied solely upon accounts given in the literature for knowledge of the behavior of mitochondria under similar conditions. Moreover, we feel that the dye should be employed in greater dilution to bring out its specificity, as the experiments which follow will show.

EXPERIMENTAL.

We have accordingly attempted to make a direct comparison of the Janus green reactions of mitochondria in living lymphocytes and of bacteria under exactly the same conditions. Lymphocytes were chosen because, unlike fixed tissue cells, they may be brought under the microscope with very little mechanical manipulation and studied with ease in the living condition. Their reactions to Janus green have also been standardized. *Bacillus proteus*, *Streptococcus hæmolyticus*, pneumococcus, *Bacillus megatherium*, *Bacterium pneumosintes*,¹³ and *Bacillus tuberculosis* (human) were selected as widely representative. They afford examples of microorganisms which are filterable and non-filterable, Gram-positive and Gram-negative, acid-resistant and -non-resistant, aerobic and anaerobic, sporulating and non-sporulating, branching and non-branching, capsulated and non-capsulated, pathogenic (for man and laboratory animals) and non-pathogenic, and which differ in morphology, so that their study may be expected to yield a comprehensive answer.

¹² Hopkins, F. G., *Nature*, 1913-14, xcii, 213.

¹³ Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.

In each case a very small amount of blood from the ear of a rabbit was placed on a slide; to this was added, with a pipette, a small drop of bacterial emulsion and a solution of Janus green B of known concentration in 0.85 per cent saline solution. The pressure of the

TABLE I.

Comparison of the Avidity with Which Mitochondria in Living Cells and Bacteria Take up Janus Green B.

Concentration of Janus green.	Mito-chondria.	<i>B. proteus.</i>	<i>Strepto-coccus hæmoly-ticus.</i>	<i>B. mega-therium.</i>	Pneumo-coccus.	<i>Bacterium pneumo-sintes.</i>	<i>B. tuber-culosis.</i>
1:1,000	+			+			
1:2,000	+			+			
1:3,000	+			+			
1:4,000	+			+			
1:5,000	+			+			
1:6,000	+			+			
1:7,000	+			+			
1:8,000	+			+			
1:9,000	+			+			
1:10,000	+	—	+	—	—	+	+
1:20,000	+		+	—		+	—
1:30,000	+		+	—		+	
1:40,000	+		+	—		—	
1:50,000	+		+	—			
1:60,000	+		+	—			
1:70,000	+		—	—			
1:80,000	+			—			
1:90,000	+			—			
1:100,000	+			—			
1:200,000	+			—			
1:300,000	+			—			
1:400,000	+			—			
1:500,000	+			—			

The method employed was first to test the reaction of *B. megatherium* to the various concentrations of the dye. Having determined that a concentration of 1:10,000 failed to stain this microorganism, we then started at this dilution with the other bacteria, continuing until a negative reaction was reached.

cover-glass brought about a sufficient mixture of fluids. By this method the reactions of living organisms and of mitochondria within living cells may easily be compared side by side and under identical conditions. The results are indicated in Table I.

It was soon found that the mitochondria in living lymphocytes surpass all the organisms examined in their affinity for Janus green. They stain intensely in dilutions of 1:100,000 and may even be colored on the addition of one part of Janus green in half a million of salt solution. The actual dilution of Janus green in contact with the cells is considerably greater than is indicated in the table, for we must take into consideration its further dilution through admixture with the blood and the bacterial emulsion. Viewed in a test-tube a column of, for example, 1:300,000 Janus green about 1 cm. in diameter shows very little color, yet when it is applied to lymphocytes the contained mitochondria are able to concentrate it in their substance so that a mitochondrial rod having an area 1 by $0.2\ \mu$ is plainly colored when observed under the oil immersion lens with strong artificial light.

It is safe to say that none of the bacteria examined are colored on the addition of Janus green in a dilution of 1:100,000. Only two—*Streptococcus hæmolyticus* and *Bacterium pneumosintes*—were stained in a concentration of less than 1:10,000. The readings vary somewhat, depending upon the relative volumes of blood, bacterial emulsion, and of Janus green. But the study of living lymphocytes with their mitochondria intensely stained surrounded by organisms which have not taken up any of the dye leaves little room for doubt that in this respect the reactions of mitochondria and bacteria are entirely different.

For a direct comparison in fixed and stained preparations, the pancreas of the rabbit was selected as a tissue in which mitochondria are well known, through many papers^{14, 15} aimed at a determination of their relation to zymogen formation. The same organisms were suspended in saline solution and injected with a hypodermic syringe into the substance of small pieces of this organ (about 3 mm. in diameter), just after its removal from the body. Immediately thereafter the fragments were fixed and stained in a variety of ways. Five rabbits were used. The pancreas of the first received *Bacillus proteus* and *Streptococcus hæmolyticus*; the second, *Bacterium pneumosintes*; the third, *Bacillus megatherium* and pneumococcus; the fourth,

¹⁴ Hoven, H., *Anat. Anz.*, 1910, xxxvii, 343.

¹⁵ Laguesse, E., *Bibliog. Anat.*, 1911, xii, 273.

Bacillus tuberculosis and *Bacterium pneumosintes*; and the fifth was used as a normal control. By this procedure each organism was brought into immediate contact with mitochondria-laden cells with very little traumatism and without the possibility of either of them being modified through a pathological process of adjustment.

Ordinary fixatives in every-day use in cytology and in bacteriology were employed, as follows: 10 per cent formalin; Zenker's fluid with and without acetic acid; 95 per cent alcohol; Giemsa's fluid (saturated aqueous corrosive sublimate 2 parts and absolute alcohol 1 part); Regaud's fluid (3 per cent potassium bichromate 4 parts and commercial formalin 1 part); A. O. B., or Bensley's acetic-osmic-bichromate mixture (2.5 per cent potassium bichromate 16 cc., 2 per cent osmic acid 4 cc., with 2 drops of acetic acid); acetic-sublimate (saturated aqueous corrosive sublimate 25 cc. and acetic acid 1.25 cc.); and Bouin's fluid (saturated aqueous picric acid 15 cc., commercial formalin 5 cc., and acetic acid 1 cc.).

All the tissues with the exception of those fixed in 95 per cent alcohol and Bouin's fluid were treated as follows: fixed over night; rinsed in water and passed to 50 per cent alcohol at 9 a.m.; 70 per cent alcohol at 10 a.m.; 95 per cent alcohol at 11 a.m.; absolute alcohol at 12 noon; half absolute alcohol and xylol at 1 p.m.; xylol at 1.30 p. m.; 60°C. paraffin at 2.30 p.m.; and embedded at 4 p.m.

After fixation in 95 per cent alcohol tissues were passed to absolute alcohol at noon and carried on as indicated, while after Bouin's fluid they were passed to absolute alcohol at 9 a.m.; half absolute alcohol and xylol at 10 a.m.; xylol at 10.30 a.m.; paraffin at 11 a.m.; and embedded at noon, or transferred to absolute alcohol at noon and embedded as indicated above.

All sections were cut 4 μ in thickness and were stained by Giemsa's method (G), with iron-hematoxylin (FeH), and with fuchsin and methyl green (FMG). The results are given in Tables II to V.

These tables show, from the number of negative signs, that the mitochondria are in general less resistant than the bacteria. For example, they are invariably destroyed by treatment in this manner with 95 per cent alcohol and with Bouin's fluid. They are occasionally and imperfectly preserved by fixation in Zenker's with and without acetic acid, in 10 per cent formalin, in sublimate-acetic, and in Giemsa's

TABLE II.

Mitochondria, B. proteus, and Streptococcus hemolyticus.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. proteus.</i>	<i>Strepto- coccus hemoly- ticus.</i>
381	10 per cent formalin.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
382	Zenker's fluid less acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
383	Giemsa's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
384	95 per cent alcohol.	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+
385	Regaud's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
386	A. O. B.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
387	Zenker's fluid with acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
388	Sublimate-acetic.	G	—	+	+
		FeH	Tr.	+	+
		FMG	—	—	—
389	Altmann's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
390	Bouin's "	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+

TABLE III.

Mitochondria and Bacterium pneumosintes.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>Bacterium pneumosintes.</i>
381	10 per cent formalin.	G	—	+
		FeH	+	+
		FMG	—	+
382	Zenker's fluid less acetic.	G	—	+
		FeH	+	+
		FMG	+	+
383	Giemsa's fluid.	G	—	+
		FeH	Tr.	+
		FMG	—	+
384	95 per cent alcohol.	G	—	+
		FeH	—	+
		FMG	—	+
385	Regaud's fluid.	G	—	+
		FeH	+	+
		FMG	+	+
386	A. O. B.	G	—	+
		FeH	+	+
		FMG	+	+
387	Zenker's fluid with acetic.	G	Tr.	+
		FeH	+	+
		FMG	+	+
388	Sublimate-acetic.	G	—	+
		FeH	—	+
		FMG	—	—
389	Altmann's fluid.	G	—	+
		FeH	+	+
		FMG	+	+
390	Bouin's "	G	—	+
		FeH	—	+
		FMG	—	+

TABLE IV.

Mitochondria, B. megatherium, and Pneumococcus.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. mega- therium.</i>	Pneumo- coccus.
401	10 per cent formalin.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
399	Zenker's fluid less acetic.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
397	Giemsa's fluid.	G	—	+	+
		FeH	Tr.	+	+
		FMG	—	+	+
403	95 per cent alcohol.	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+
400	Regaud's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
395	A. O. B.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
398	Zenker's fluid with acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
402	Sublimate-acetic.	G	—	+	+
		FeH	—	+	+
		FMG	—	—	—
396	Altmann's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
404	Bouin's "	G	—	+	+
		FeH	—	+	+
		FMG	—	+	Faint.

TABLE V.

Mitochondria, B. tuberculosis, and Bacterium pneumosintes.

Tissue No.	Fixation.	Stain.	Mito- chondria.	<i>B. tuber- culosis.</i>	<i>Bacterium pneumo- sintes.</i>
412	10 per cent formalin.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
410	Zenker's fluid less acetic.	G	—	+	+
		FeH	+	+	+
		FMG	—	+	+
408	Giemsa's fluid.	G	—	+	+
		FeH	Tr.	+	+
		FMG	—	+	+
405	95 per cent alcohol.	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+
411	Regaud's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
406	A. O. B.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
409	Zenker's fluid with acetic.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
413	Sublimate-acetic.	G	—	+	+
		FeH	—	+	+
		FMG	—	—	—
407	Altmann's fluid.	G	—	+	+
		FeH	+	+	+
		FMG	+	+	+
414	Bouin's "	G	—	+	+
		FeH	—	+	+
		FMG	—	+	+

mixtures. This persistence after fixation in Zenker's fluid with acetic acid and in sublimate-acetic is unusual and may be due to the rapidity of dehydration. As one would expect, Regaud's fluid, A. O. B., and Altmann's fluid proved to be the best preservatives for mitochondria. The bacteria, on the other hand, resist the action of all of the ten fluids. In the preparations the distinction between the solubility of mitochondria and bacteria is often very sharp. In a single field bacteria may be seen in close contact with cells completely deprived of mitochondria (by alcohol and Bouin's fluid) although both bacteria and mitochondria have been subjected to precisely the same influences.

There is also a pronounced difference in staining reaction. Although iron-hematoxylin and fuchsin and methyl green stain both bacteria and mitochondria, Giemsa's stain, which is perhaps the best adapted for the demonstration of bacteria, colors the mitochondria little, if at all. The failure of this so called nuclear dye to color mitochondria specifically points to a difference which we must consider to be fundamental between mitochondria and bacteria. The staining of bacteria with basic dyes is usually attributed to the presence in them of chromatin-like materials containing iron. Molisch,¹⁶ particularly, has made many contributions regarding the widespread occurrence of iron in bacteria. But Nicholson¹⁷ has applied the Macallum¹⁸ hematoxylin and hydrochloric acid test for iron to tissues known to contain mitochondria without obtaining any trace of coloration, which is strong evidence of the absence of masked iron in mitochondria.

In respect to their morphology also we feel that our preparations show a noticeable difference between mitochondria and bacteria. While not denying that some types of bacteria exhibit a high degree of pleomorphism and that, in searching through the body, cells may be found in which the mitochondria are much more uniform in appearance than in the pancreas, we refer to the observations of Lewis and Lewis¹⁹ on mitochondria in tissue cultures. These investigators followed

¹⁶ Molisch, H., *Die Pflanze in ihren Beziehungen zum Eisen*, Jena, 1892.

¹⁷ Nicholson, F. M., personal communication.

¹⁸ Macallum, A. B., *Quart. J. Micr. Sc.*, 1896, xxxviii, 175.

¹⁹ Lewis, M. R., and Lewis, W. H., *Am. J. Anat.*, 1914-15, xvii, 339.

the changes in form of mitochondria in living cells and succeeded in demonstrating a plasticity and modifiability far greater than that possessed by any organism with which we are familiar.

DISCUSSION.

From the foregoing experiments it appears that mitochondria show microchemical and tinctorial properties differing from those of bacteria. It seems pertinent, however, to give a definition of bacteria and to indicate the points on which definite information is required before the theories advanced by Altmann, Portier, and Wallin can be considered acceptable.

According to Park and Williams:²⁰ "The properties of bacteria . . . which are fairly constant under uniform conditions and which have been more or less used in systems of classification, are those of spore and capsule formation, motility (flagella formation), reaction to staining reagents; relation to temperature, to oxygen and to other food material, and, finally, their relation to fermentation and disease." To which we may add the property of forming smaller or larger aggregates (colonies), the individuals of which are, however, physiologically independent (Zinsser²¹), and the development of characteristic modes of growth on suitable artificial media.

It must be admitted in all these respects that a great gulf remains between mitochondria and bacteria. In respect to mitochondria definite spores and capsules have not been noted. The mitochondrial blebs which Wallin suggests may be due to fixation bear in our judgment no resemblance to spores. They often contain easily recognizable substances like neutral fat or starch (in plants) or a clear watery chromophobe fluid, and may clearly be seen in living acinus cells of the pancreas. Motility due to flagellar action has not been observed, and certain differences in staining reaction have been noted above. The temperature, oxygen, and food requirements of mitochondria can only be expressed in terms of the requirements necessary for the vitality of the cell as a whole. It seems unsafe to conclude, as Wallin

²⁰ Park, W. H., and Williams, A. W., *Pathogenic microorganisms*, Philadelphia and New York, 7th edition, 1920, 24.

²¹ Zinsser, H., *A textbook of bacteriology*, New York, 5th edition, 1922, 9.

has done, that because certain forms of bacteria cannot be demonstrated by staining after treatment with moist heat at 49°C. for 30 minutes they necessarily resemble mitochondria which are also destroyed by somewhat similar treatment, because many bacteria are much more resistant. No mitochondria, on the other hand, are known to resist a temperature of over 50°C. Neither is there any parallelism between mitochondria and bacteria in their relation to fermentation and to disease. In fact, their tendency is rather to decrease than to increase in diseases of infectious nature. Furthermore, there is no reason to believe that mitochondria possess the power of independent and characteristic growth apart from cells. Experiments in this laboratory²² on the cultivation of kidney, brain, spleen, heart muscle cells, and blood in a variety of fluid and semi-solid media by aerobic and anaerobic methods have failed to reveal the multiplication of any forms at all suggestive of mitochondria.

The suggestion that mitochondria are independent microorganisms rests in our judgment upon no other evidence than a slight similarity in form of substances of about the same size.

CONCLUSION.

A direct comparison of mitochondria and bacteria in the living condition, as well as in permanent preparations, subjected to the same influences, and viewed side by side, reveals microchemical and tinctorial differences which can only be attributed to a fundamental dissimilarity in their chemical constitution.

²² Reported in part by Olitsky (Olitsky, P. K., *J. Exp. Med.*, 1921, **xxxiv**, 525).

INSUSCEPTIBILITY TO SENSITIZATION AND ANAPHYLACTIC SHOCK.

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(Received for publication, July 10, 1922.)

The object of the experiments that are to be described was to gain some information concerning the factors which determine the variations that species and individuals show towards certain forms of intoxication. The study forms a continuation of some investigations previously made with Mackenzie (1) and later completed by Mackenzie and Leake (2). In these previous investigations the immunological processes involved in the production of serum disease in the human being were selected as the problem.

It is well known that the incidence of serum disease following the injection of therapeutic sera increases roughly in proportion to the amount of serum injected, so that up to a certain point the susceptibility to serum disease is dependent upon the size of the dose. After a dose of approximately 75 to 100 cc. of serum is reached the incidence of serum disease remains about stationary at 80 to 90 per cent, and no matter how much more serum is administered, a small proportion of individuals always escapes the manifestations of serum sickness.

Recently, Coca, Deibert, and Menger (3) have shown, moreover, that the North American Indian is relatively much less susceptible than the white race, for they state that of twenty-six healthy Indians each of whom received 100 cc. of normal horse serum intravenously, only twelve, or 46 per cent, developed symptoms of serum sickness, and in most of these instances the disease ran a short and very mild course, averaging only 2 days.

It was desired in the previous experiments to study the protective mechanism possessed by the small group of white patients that escaped serum sickness. These individuals were found to differ from the susceptible inasmuch as they did not produce demonstrable antibodies in the form of precipitins and anaphylactic antibody, and inas-

much as the antigen, or horse serum, remained in the circulation as an apparently innocuous substance for periods of weeks or months after it was injected. In the susceptible individuals precipitins were usually formed in large amounts, and the antigen disappeared rapidly from the circulation with the subsidence of serum disease. It seemed, therefore, that the insusceptible individual possessed some mechanism which inhibited the union of antigen with the cells of the body and thus prevented the effects which follow the rapid and usual reactions between the foreign protein and the tissues of the host. According to Metchnikoff, a similar form of mechanism explains the insusceptibility of certain cold blooded animals to diphtheria and tetanus toxin. And according to Coca, Russell, and Baughman (4) the partial impermeability of the cells of the rat to diphtheria toxin explains their great resistance to this poison.

Individual variation to sensitization by foreign proteins is known to occur in all laboratory animals but in the guinea pig or even the rabbit, it is so rare that experiments with these animals seemed unprofitable.

The white rat, on the other hand, has generally been considered as an animal which could not be made anaphylactic (Doerr (5)). It is not possible, however, to find many observations in the literature which afford much information on this subject.

Arthus (6) simply states that he has observed anaphylactic phenomena in white rats after repeated injections of foreign sera. Rosenau and Anderson (7) mentioned the fact that they had tested the anaphylactic reaction in monkeys, rabbits, mice, dogs, cats, rats, chickens, and pigeons and stated that they obtained positive results with the dog, rabbit, and cat. Uhlenhuth, Haendel, and Steffenhagen (8) reported that they were unable to produce anaphylactic shock in white rats or mice. These observations were confirmed by Trommsdorff (9), who failed in attempts to sensitize twenty-five white rats and twenty-five mice to egg white or to horse serum. The second injection of these proteins was administered intravenously after intervals of 5 days to 12 weeks. But the most complete study of this subject has probably been made by Novy and De Kruif (10), who, in attempting to sensitize white rats to horse serum, beef serum, rabbit serum, and egg white, found that the second injection made intravenously of such large amounts of the undiluted antigens as 2 to 4 cc. in these supposedly sensitized rats, did not produce any symptoms which could definitely be considered as anaphylactic, and was never fatal. When, on the other hand, antigens diluted with distilled water were given intravenously in amounts of

7 to 10 cc. at the second injection, violent symptoms occurred and in many animals were followed by death. In the normal rat similar amounts of the mixture of antigen and distilled water produced little untoward effect. The intoxication brought about in the supposedly sensitized rat, by the second injection of diluted antigen, proved, however, to be non-specific, for it could be produced quite as readily by the injection of an equal amount of distilled water without antigen. Novy and De Kruif concluded, therefore, that though the rat was practically insusceptible to specific anaphylactic shock, the repeated injection of a foreign protein, nevertheless, produced some change which rendered the animal highly sensitive to the intravenous injection of distilled water and gave rise to a state which was conducive as well to a much more rapid formation of anaphylatoxin in the serum of the shed blood than could be obtained with normal rat serum. Novy and De Kruif (11) also studied the effect upon white rats of the intravenous injection of anaphylatoxin, agar sol-gel, and peptone. To all of these substances the rats were much more refractory than guinea pigs. Weight for weight, the rat tolerated 100 times as much anaphylatoxin as guinea pigs, three times as much agar sol-gel, and about seven times as much 10 per cent peptone solution. For white rats the lethal dose of 10 per cent solution of Witte's peptone was 2 gm. per kilo.

From these experiments it seems evident that the white rat is both highly refractory to anaphylaxis and very resistant to the action of such toxic substances as anaphylatoxin and peptone.

EXPERIMENTAL.

In the present investigation the following plan of procedure was adopted: (1) study of the symptoms produced in white rats by intravenous injection of peptone and histamine; (2) attempts to sensitize white rats to horse serum; (3) determination of the state of sensitization (*a*) by intravenous and subdural injection of antigen, (*b*) by skin reactions, and (*c*) by the uterine strip reaction; (4) attempts to sensitize passively guinea pigs with the serum of white rats immunized to horse serum; and (5) study of the antigen-antibody content of the serum of white rats injected with horse serum.

Symptoms Produced by the Intravenous Injection of Peptone and Histamine.

In order to obtain some criteria of shock similar to that in anaphylaxis, white rats weighing approximately 100 to 125 gm. were given intravenous injections of solutions of Witte's peptone and of histamine.

1 gm. of Witte's peptone was dissolved by heat in distilled water, centrifuged, and the supernatant fluid employed for injection. The histamine was made up in 1 and 2 per cent solution in 0.85 per cent NaCl.

The intravenous injection of 0.1 gm. of peptone per 100 gm. of body weight caused very severe symptoms. Immediately after the injection the animals went into collapse. They lay flat upon the table with all four legs spread out; the respirations were slow and difficult. After 2 to 3 minutes they often made an attempt to stand but failed; within 4 or 5 minutes there were sometimes slight convulsive movements with irregular gasping respirations; in about 10 minutes the respirations would become rapid (68 in one animal), while the paralysis and spasmodic convulsive movements continued. One animal recovered after this dose at the end of 2 hours. A second animal died in 10 minutes.

The effects following the intravenous injection of histamine were quite similar; immediate collapse, gasping respiration, paralysis, convulsive seizures, fall in temperature, the expulsion of feces and urine, and the discharge of fluid from the nose were all prominent features. One noteworthy symptom was an extreme and acute exophthalmos. In those animals that recovered, the severe symptoms persisted for an hour or longer. Two rats withstood a dose of 10 mg. of histamine per 100 gm. of body weight, two animals 15 mg. of histamine per 100 gm. of body weight, and one animal died after receiving 30 mg. of histamine per 100 gm. of body weight.

Attempts to Sensitize to Horse Serum.

A criterion having been established for symptoms which by analogy with other animals should simulate anaphylaxis in the white rat, attempts were made to sensitize white rats by repeated injections of horse serum given subcutaneously, intraperitoneally, intravenously, or subdurally. Twenty-seven experiments in twenty-three rats weighing from 75 to 150 gm. were devised for this purpose. Thirteen rats were given three injections of from 0.1 to 0.5 cc. of horse serum subcutaneously or intraperitoneally at 2 to 3 day intervals, three rats six injections of similar amounts, two rats a single injection of 0.5 cc. intravenously, and five rats three injections subcutaneously or intra-

TABLE I.

Rat No.	Weight.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last injection.	Amount of horse serum.	Method.	Symptoms.
	gm.	1921	cc.		days	cc.		
1	85	Oct. 5	0.5	Subcutaneous.	21	2.5	Intraperitoneal.	None.
		" 7	0.5	"				
		" 10	0.5	"				
2	84	" 5	0.5	"	22	0.5	Intravenous.	"
		" 7	0.5	"				
		" 10	0.5	"				
4	94	" 6	0.5	Intraperitoneal.	21	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"				
6	83	" 5	0.5	"	22	0.5	"	"
		" 7	0.5	"				
		" 10	0.5	"	(Jan. 5, 1922.) 66	1.0	"	"
7	154	" 17	0.1	Subcutaneous.	29	0.6	"	"
		" 20	0.25	"				
		" 24	0.25	"				
8	138	" 17	0.1	"	29	0.5	"	No definite symptoms. Remains quiet; respirations a trifle irregular.
		" 20	0.25	"				
		" 24	0.25	"				

TABLE I—Continued.

Rat No.	Weight. gm.	Date sensitized.	Amounts of horse serum.		Method.	Length of time after last injection. days	Amount of horse serum.	Method.	Symptoms.
			1921	cc.					
11	136.5	Oct. 17		0.1	Intraperitoneal.	29	0.5	Intravenous.	None.
		" 20		0.25	"				
		" 24		0.25	"				
13	77	" 24		0.2	"	31	1.0	"	"
		" 26		0.4	"				
		" 28		0.5	"				
14	68	" 24		0.2	Subcutaneous.	31	0.5	"	Lies flat 5 to 10 min. after injection.
		" 26		0.4	"				
		" 28		0.5	"				
15	106	" 24		0.2	"	31	0.8	"	Lies flat 5 min. after injection. Seems weak. 15 min., recovery.
		" 26		0.4	"				
		" 28		0.5	"				
15	150	Nov. 29		0.8	Intravenous.	38	1.0	"	None.
16	83	Oct. 24		0.25	Intraperitoneal.	31	1.0	"	"
		" 26		0.5	"				
		" 28		0.5	"				
17	93	" 24		0.2	"	31	1.0	"	"
		" 26		0.5	"				
		" 28		0.5	"				

18	89	Oct. 24	0.2	Intraperitoneal.	31	1.0	Intravenous.	None
		" 26	0.5	"				
		" 28	0.5	"				
19	98	Nov. 2	0.5	Intravenous.	21	0.5	"	"
					44	0.5	Intraperitoneal.	"
20	150	" 2	0.5	"	21	0.5	Intravenous.	"
					44	0.5	Intraperitoneal.	"
33	92	1922 Jan. 5	0.2	"	27	1.0	Intravenous.	"
		" 9	0.5	Intraperitoneal.				
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
41	125	" 6	0.2	"	27	1.0	"	Seems slightly weak but walks well. No respira- tory difficulty.
		" 9	0.5	"				
		" 11	0.5	"				
		" 13	0.5	"				
		" 16	0.5	"				
		" 18	0.5	"				
Controls.					1921			
21	127	Nov. 28	1.0				"	None.
22	78	" 28	1.0				"	"
23	140	" 28	1.0				"	"
19	98	" 2	0.5				"	"
20	150	" 2	0.5				"	"

TABLE II.

Rat No.	Weight. gm.	Date sensitized.	Amounts of horse serum.	Method.	Length of time after last in- jection.	Amount of horse serum.	Method.	Symptoms.
		1921	cc.		days	cc.		
13	74	Oct. 24	0.2	Subcutaneous.	38	0.2	Subdural.	General convulsion; rapid respirations. 4 min., weak. 10 min., still collapsed; weak on left side. 15 min., recovered but paralyzed on left side.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
14	68	Oct. 24	0.2	Subcutaneous.	38	0.2	"	None.
		" 26	0.4	"				
		" 28	0.5	"				
		Nov. 28	0.5	Intravenous.				
16	75	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Convulsions; stops breathing. 2 min., recovered. 3 min., gasping. 10 min., dyspnea continued. 1 hr., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
17	100	Oct. 24	0.2	Intraperitoneal.	38	0.5	"	Moderate collapse; stops breathing. Respiration started in 1 min. 3 min., crawls; respirations rapid. 6 min., walks about recovering.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				
18	105	Oct. 24	0.2	Intraperitoneal.	38	0.3	"	Immediate collapse. Respiration starts in 1 min. 2 min., deep breathing. 3 min., labored respirations. 7 min., stands; respirations difficult. 18 min., recovered.
		" 26	0.5	"				
		" 28	0.5	"				
		Nov. 28	1.0	Intravenous.				

42	120	1922 Jan. 6	0.2	Intraperitoneal. “	27	0.2	Subdural.	Walks about; respirations rapid and slightly difficult. Recovered in 3 min.
Controls. 32	105	“ 9	0.5	“				
		“ 11	0.5	“				
		“ 13	0.5	“				
		“ 16	0.5	“				
		“ 18	0.5	“				
33	92					0.5	“	Immediate collapse; convulsions. 2 min., lies on side; hind leg weak. 7 min., better. 15 min., recovery.
						0.2	“	Immediate convulsions; respiration stopped. 2 min., rapid respirations; recovery. 10 min., recovered.
34	105							
						0.5	“	Immediate shivering; respiration ceases. 2 min., breathing. 3 min., respirations difficult. 6 min., still very sick. 7 min., recovery.
60	115							
						0.2	“	Immediate collapse; respirations rapid; sneezing; coughing. 6 min., respirations difficult; fluid from nose. 10 min., sneezing; respirations difficult. 30 min., still very sick. 1 hr., recovery.

peritoneally and 1 month later 1 cc. intravenously. The test injections were made from 21 to 44 days after the last sensitizing dose. These experiments are recorded in Tables I and II. In seventeen experiments the injections of 0.5 to 1 cc. of horse serum into the femoral vein of these supposedly sensitized rats (Table I) seemed quite harmless and never produced any symptoms that resembled in the least those obtained with peptone or histamine, much less death. When injections of from 0.2 to 0.5 cc. of horse serum were made subdurally in six rats, immediate and violent symptoms usually occurred, which, however, never proved fatal, and exactly similar symptoms were obtained in the normal control rats (Table II).

From these experiments one must conclude that anaphylaxis to horse serum cannot be obtained in white rats by a second injection of antigen given intravenously or subdurally.

Determination of the State of Sensitization.

The skin of seventeen white rats subjected to repeated injections of horse serum given subcutaneously, intraperitoneally, or intravenously was tested for a specific reaction on the 20th to the 63rd day after the last injection of antigen. The sides of the rat were shaved 2 or 3 days preceding the experiment and an injection of 0.02 cc. of horse serum diluted 1:10 with 0.85 per cent NaCl was made intracutaneously. As controls, similar amounts of rabbit serum diluted 1:10, or of 0.85 per cent NaCl, were given intracutaneously to the inoculated rats and at the same time both horse serum and rabbit serum in similar amounts and dilutions were injected intracutaneously into normal rats. The skin of the rats was observed every 15 minutes for a period of 1 to 2 hours and again at the end of 12, 24, and after 48 hours. In no instance was there any evidence of a skin reaction such as may be obtained in the guinea pig, rabbit, or in man, and in no instance did the test injection produce an effect which differed from that in the controls.

It must be concluded, therefore, that the skin of the white rat subjected to repeated injections of horse serum does not differ from that of the normal rat in its reaction to subsequent intracutaneous injections of horse serum in the amounts utilized.

It is generally assumed that the reactions of the smooth muscle of the sensitized virgin guinea pig as employed by Dale is one of the most delicate tests for sensitization; and for this reason six virgin white rats averaging 50 to 60 gm. were injected intraperitoneally with 0.2, 0.2, 0.5, and 0.5 cc. of horse serum every other day, and after intervals of from 38 to 54 days the uteri were removed, suspended in 250 cc. of oxygenated Locke's solution at 39°C., and tested for their reaction to horse serum. As control antigens, rat serum, rabbit serum, and sheep serum were employed. In five control experiments with the uteri of normal virgin rats, it was found that horse serum in doses of from 1 to 2.5 cc. produced no change in the regular rhythmic uterine contraction but sometimes resulted in a very slight general shortening of the muscle strip which was made evident by an elevation of the line of contractions upon the surface of the drum. The effect of histamine in doses of 1 mg. was to produce immediate relaxation of the muscle strip with great slowing of the contraction, which often became irregular.

The effect of horse serum on the uteri of the inoculated rats in doses of 1 cc. did not differ from that produced by horse serum upon the uteri of normal rats. No unusual contractions, such as are seen in the guinea pig uterus, were observed, and in no instance was the relaxing effect noted with histamine encountered.

It was therefore concluded that the uteri of white rats receiving repeated injections of horse serum did not differ in their reaction to horse serum from those of normal rats.

It seemed possible to conclude, therefore, from these series of experiments that white rats could not be made anaphylactic to horse serum, as tested by several methods. The next step was to determine upon what this refractory condition depended.

This insusceptibility might be explained by at least one of two hypotheses: first, that the tissues of the rat were insusceptible to the poisonous effect which follows the reaction presumably of antibody and antigen in the highly sensitized animal when a second injection of antigen is administered; or, second, that the tissues of the rat were for some reason unable to form an hypothetical anaphylactic antibody or were in some way prevented from accomplishing this.

To answer the first question, experiments were done to determine whether the serum of supposedly immunized rats contained anaphylactin and was capable of transferring passive anaphylaxis to guinea pigs.

Attempts to Sensitize Passively Guinea Pigs with the Serum of White Rats Immunized to Horse Serum.

The serum of fourteen white rats, subjected to repeated intraperitoneal injections of horse serum, was injected within a few hours of bleeding into guinea pigs. Seven guinea pigs received from 0.4 to 0.6 cc. of rat serum intravenously and seven from 0.75 to 1 cc. intraperitoneally. After an interval of 23 to 25 hours these guinea pigs were given 0.5 cc. of horse serum intravenously. No symptoms which could be interpreted as those of anaphylactic shock were observed in any instance. The experiments are summarized in Table III.

These experiments furnish a strong argument, in spite of the resistance of the white rat to peptone and histamine, against the idea that the rat behaves immunologically in the same manner as the guinea pig, rabbit, and dog, but nevertheless escapes the symptoms of anaphylaxis owing to its resistance to the poisonous effects of the shock. It was, consequently, necessary to seek further for an explanation of its insusceptibility to anaphylaxis.

Antigen-Antibody Content of the Serum of White Rats Injected with Horse Serum.

Experiments were therefore instituted to determine whether the second hypothesis was correct and to discover if the same protective mechanism towards sensitization existed in the rat as has been described for the human being. For this purpose a study of the antigen content of the serum of rats injected with horse serum was made. Twenty-one rats were used. Since the size of the rat prohibited the use of a single rat for repeated bleedings, it was necessary to use a single rat for each experiment. The rats, therefore, were killed by bleeding from the heart at various times after a single intravenous injection of approximately 1 cc. of horse serum per 100 gm. of body weight. By employing the precipitin reaction in the manner described

TABLE III.

Guinea pig No.	Weight. gm.	Date.	Rat serum No.	Amount of rat serum. cc.	Method.	Inter-val. hrs.	Amount of horse serum injected intravenously. cc.	Symptoms.
6	240	1922 Jan. 13	8	1.0	Intraperitoneal.	23	0.5	None.
5	200	" 13	15	0.6	Intravenous.	23	0.5	Shivering; no other symptoms.
10	230	" 19	19	0.5	"	24	0.5	None.
11	240	" 19	20	0.5	"	24	0.5	"
12	180	" 20	35	0.5	"	23	0.5	"
13	190	" 20	36	0.5	"	23	0.5	Shivering; no other symptoms.
14	200	Feb. 1	37	0.4	"	23	0.5	None.
15	225	" 1	38	0.5	"	23	0.5	"
19	150	1919 Mar. 8	33	0.75	Intraperitoneal.	24	0.5	Shivering; slight weakness of hind legs. Recovered in 3 min.
20	111½	" 8	34	1.0	"	24	0.5	None.
21	146	" 8	41	1.0	"	24	0.5	"
22	136	" 8	42	1.0	"	24	0.5	Slight shivering; slight weakness; immediate recovery.
23	120	" 8	59	0.2	"	25	0.5	Shivering.
24	223	" 8	60	1.0	"	25	0.5	None.
Controls.								
8	250	Jan. 13					0.5	"
7	230	" 13			Intraperitoneal.	23	0.5	"
9	250	" 20	Normal.	0.8	Intravenous.	48	0.5	"
17	190	Feb. 1	"	0.5	"	23	0.5	Shivering.
25	120	Mar. 8	"	1.0	Intraperitoneal.	24	0.5	None.
1	220	Jan. 19	Jan. 6. Sensitized to horse serum.			13	0.5	Immediate symptoms; death in 2 min.
2	230	Feb. 1	" 6. "	6.	"	25	0.5	" " " 3 "
3	220	Mar. 8	" 6. "	6.	"	61	0.5	Death in 2 min.

in previous communications—except that all sera were employed in amounts of 0.25 cc.—it could be shown that antigen (horse serum) when injected intravenously did not persist in the circulation but disappeared in from 12 to 14 days, a period which is approximately that required by the rabbit to dispense with a similar antigen.

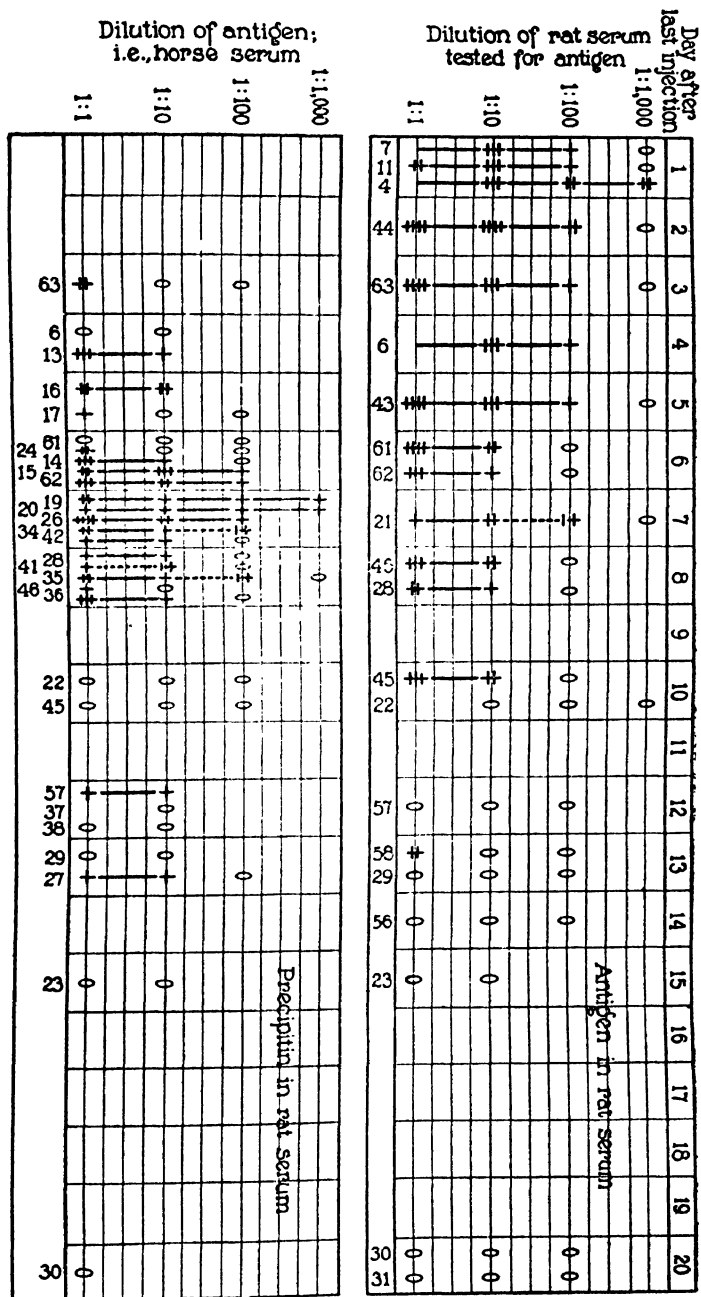
It was thought that the kidney of the rat might be permeable to the horse serum and thus allow of its escape from the body, but precipitin reactions upon the urine of white rats injected with horse serum failed to demonstrate antigen in the urine from the time of injection to the time of the disappearance of the horse serum from the circulation.

It was necessary, therefore, to search for some other explanation. Although it seemed *a priori* improbable that the white rat which was refractory to sensitization would form precipitins, still tests were made for specific precipitins in the serum of twenty-eight white rats receiving single and repeated injections of horse serum, and it was found that these animals were capable of forming such precipitins in fairly high concentration. Precipitins for horse serum could be demonstrated in the serum of twenty of the twenty-eight rats, appearing first on the 3rd to the 4th day after the last of a series of inoculations and increasing in intensity up to the 6th or 8th day, when they were present with great regularity. After this time, there was a rapid decline in positive reactions, for the sera from only two of nine rats drawn after the 10th day showed precipitins. It was also found that it was during the high concentration of precipitins in the serum that the antigen disappeared from the circulation. A graphic presentation of the precipitinogen-precipitin balance is shown in Text-fig. 1.

The mechanism of precipitin formation and the relationship between precipitin and precipitinogen (horse serum) proved, therefore, to be much the same as it is in the rabbit.

Thus, though sensitization to horse serum did not occur, the cells of the white rat had apparently an avidity for horse serum which was as great as, or even greater than that of the rabbit.

Though there has been considerable discussion as to the identity of the anaphylactic antibody and precipitin, the work of Doerr and Russ (12) in rabbits and the subsequent observations of Weil (13) have lent such strong support to the idea that these two bodies are one and the same, that most investigators (Doerr (5), Zinsser (14), Wells (15))



have accepted this explanation as the correct one. Doerr in particular, after a complete review of the literature, has come to this conclusion. It has, however, been generally known that the ease with which an animal may be sensitized to foreign proteins and the readiness with which it forms specific precipitins are not always parallel. The guinea pig, for instance, is highly subject to sensitization but is an extremely poor precipitin producer, whereas the rabbit, which is much more difficult to sensitize, produces precipitins in large amounts.

The present experiments seem to demonstrate that in white rats precipitins and anaphylactin are not identical and that good precipitin formation can occur without anaphylactic sensitization and without the appearance of anaphylactic antibody in the serum of the rat.

The differences which several species of animals show in the ease with which they may be sensitized to foreign proteins and the readiness with which they may be subjected to shock by a second injection of the same antigen have already been alluded to, and it now seems from these experiments that the rat stands at one end of the scale, as a completely refractory animal, and probably the guinea pig at the other, as representing the most highly susceptible animal. This refractory condition of the rat does not seem to be due to a resistance of specialized tissues to the toxic effect of anaphylactic shock, or to an inability of the tissues to absorb antigen, for on the one hand, the animal may be killed by histamine or peptone in large doses, and on the other, the tissues are capable of producing precipitins in considerable concentration but not anaphylactin. The explanation seems rather to be found in the fact that the preliminary injections of antigen, though capable of calling forth precipitins, fail to prepare the cells of the body in the manner necessary to make them vulnerable either by an antibody-antigen reaction or by some other mechanism to the second injection of antigen.

SUMMARY.

1. Attempts to produce anaphylactic shock in white rats by second intravenous or subdural injections of horse serum have failed.
2. It was impossible to demonstrate either by skin reactions or by the uterine reaction that white rats can be sensitized to horse serum.

3. It was not possible to sensitize guinea pigs passively with the serum of white rats presumably immunized to horse serum.

4. In spite of the fact that the white rat could not be made anaphylactic to horse serum, the tissues of the animal reacted with the horse serum to form precipitins in fair concentration and the antigen disappeared from the circulation soon after the precipitins reached their greatest concentration in the blood.

5. These experiments would indicate that in the white rat anaphylaxis and precipitin formation are independent and represent different types of immunological process.

BIBLIOGRAPHY.

1. Longcope, W. T., and Mackenzie, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1919-20, xvii, 133.
2. Mackenzie, G. M., and Leake, W., *J. Exp. Med.*, 1921, xxxiii, 601.
3. Coca, A. F., Deibert, O., and Menger, E. F., *J. Immunol.*, 1922, vii, 201.
4. Coca, A. F., Russell, E. F., and Baughman, W. H., *J. Immunol.*, 1921, vi, 387.
5. Doerr, R., *Ergebn. Hyg., Bakt., Immunitätsforsch. u. exp. Therap.*, 1922, v, 71.
6. Arthus, M., *Compt. rend. Soc. biol.*, 1903, lv, 819.
7. Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H., No. 29*, 1906, 57.
8. Uhlenhuth, Haendel, and Steffenhagen, *Centr. Bakt., 1te Abt., Ref.*, 1910, xlvii, suppl., 158.
9. Trommsdorff, R., *Arb. k. Gsndtsamte*, 1909, xxxii, 560.
10. Novy, F. G., and De Kruif, P. H., *J. Infect. Dis.*, 1917, xx, 776.
11. Novy, F. G., and De Kruif, P. H., *J. Infect. Dis.*, 1917, xx, 618, 629. Novy, F. G., De Kruif, P. H., and Novy, F. O., *J. Infect. Dis.*, 1917, xx, 657.
12. Doerr, R., and Russ, V. K., *Z. Immunitätsforsch., Orig.*, 1909, iii, 181, 706.
13. Weil, R., *J. Immunol.*, 1916, i, 19, 35, 47.
14. Zinsser, H., *Infection and resistance*, New York, 1918.
15. Wells, H. G., *Physiol. Rev.*, 1921, i, 44.

THE SUPRAVITAL STAINING OF VACCINE BODIES.

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PLATES 58 AND 59.

(Received for publication, June 26, 1922.)

INTRODUCTION.

To determine the nature of the conspicuous cell granulations found in the lesions of vaccinia is a problem in cytology of unusual interest which has continued to hold the attention of investigators.¹ They seem to represent the visible expression of a fundamental cellular reaction. From the historical point of view three lines of thought may be recognized in the development of our present knowledge of the subject, which, in its broader aspects, cannot well be considered apart from other diseases of unknown etiology.

1. On their discovery, the granulations were first named after their observers, Guarnieri's bodies in vaccinia and variola, Negri's bodies in hydrophobia, von Prowazek's bodies in trachoma, Mallory's bodies in scarlet fever, etc. Influenced, perhaps, by contemporaneous studies in malaria and other protozoal diseases, investigators have described complicated life histories based partly upon direct observations and partly upon analogy. The granules were hailed more or less tentatively as the actual parasitic causes of the diseases, were grouped among the protozoa, and were given generic and specific names, of which *Cytoryctes vaccinia* in vaccinia, and *Neurocytes hydrophobia* in hydrophobia will serve as examples.

2. But closer and repeated scrutiny of the life histories did not turn out to be reassuring, and led investigators to question whether a real step had been made in advance. A reaction set in and several papers appeared showing most clearly that in some cases at least the granules are not cell parasites but deposits of pathologic nature. By common consent the protozoal terminology was abandoned and the names of the discoverers reinstated.

3. Faced by this association of more or less specific granulations with certain infectious diseases, the organisms of which have not been seen and remain almost

¹ The early literature is reviewed by Councilman, Magrath, and Brinckerhoff (1904), by Calkins (1904), by Paschen (1911), and by von Prowazek (1912).

wholly unknown, von Prowazek (1907) offered a compromise hypothesis according to which the cytoplasmic granules are of dual nature, consisting of microorganisms embedded in a substance of nucleolar origin produced by the cell in response to their action. Von Prowazek has proposed that these organisms be grouped in a new class which he has called Chlamydozoa—literally, mantle animals—to indicate this habit of being clothed in a mantle of cellular material. In his opinion they are characteristically intracellular parasites and differ from bacteria in their method of multiplication as well as in other respects. He conceived their life history to be somewhat as follows: In the stage of elementary corpuscles they are extracellular and filterable; soon after penetration through the cell membrane they may be detected in the form of tiny initial bodies. These become coated with cellular material, grow, mature, and break down with the liberation of large numbers of the original elementary corpuscles, which, in turn, enter and infect neighboring cells with a repetition of the process.

Lipschütz (1912) has devised the term Strongyloplasmata to indicate organisms endowed with much the same attributes. He groups (1921) under this heading the viruses of molluscum contagiosum, bird-pox, sheep-pox, vaccinia, scarlet fever, verruga peruviana, herpes (zoster, genitalis, and febrilis), varicella, variola, and paravaccinia, and is supported in his contention regarding vaccinia by Gins (1922) and other recent investigators.

It seems almost to have become the fashion to classify organisms about which very little is known in one or the other of these two groups. For example, during the war the ravages of typhus and trench fever demanded very close attention in both camps, which contributed directly to our knowledge of organisms which appear to be intimately concerned in the etiology not only of these two diseases but also of Rocky Mountain spotted fever and perhaps of tsutsugamushi disease, or Japanese river sickness. They have been called *Rickettsia* in honor of Ricketts who lost his life in Mexico in 1910 while investigating their behavior in typhus, but the term is unfortunate because it carries with it the suggestion that the organisms differ from ordinary bacteria and form a group exhibiting certain characteristics in common. Although it has not been possible to prove that during some phase of their life cycle they are of dual nature (consisting in part of cellular substance), they have been referred to the Chlamydozoa by da Rocha-Lima (1916), Jungmann (1919), and others.

Looking through the recent literature one finds the opinion repeatedly expressed that real advances in our knowledge of vaccinia will only be made with the help of new methods of technique.

Steinhardt and his associates (1913, 1914) and Harde (1921) have invoked the methods of tissue culture and have succeeded in obtaining a definite multiplication of the virus *in vitro* but have not been able to concentrate it greatly or to determine its relation to vaccine bodies.

What appears to be a new avenue of approach to the study of infectious diseases of unknown etiology is contributed by MacCallum and Oppenheimer (1922), who so concentrated the virus of vaccinia, through the method of differential centrifugation, that they have been able to observe directly certain granules, which may be the infective agents, under dark-field illumination and with ordinary powers of the microscope. "They occur singly or in small groups, or sometimes in tiny beaded chains. . . . They stain faintly with Löffler's methylene blue, are gram negative, do not stain with neutral red or trypan blue, stain blue or red with Wright's stain, and take a fairly deep stain with carbolfuchsin." The authors believe that: "these granules are apparently identical with those seen in tissues and smears from vaccinia and smallpox by Prowazek, Paschen, Hallenberger, and others." A similar method is advised by Ségal (1922) for concentrating the virus of typhus fever.

A large supply² of tissues from vaccinated rabbits in various stages of the reaction has given me an opportunity to experiment widely in technique and thus to make a cytologic study of the cornea with three definite objects in view; namely, (1) to trace the origin of the material of which the vaccine bodies are constituted, (2) to ascertain whether it is in part of chlamydozoal nature, and (3) to determine how closely it is related to the granules described by MacCallum and Oppenheimer in vaccine lymph.

OBSERVATIONS.

If the edge of a sharp scalpel is passed over a typical corneal lesion and the cells thus obtained are immersed in physiological salt solution and examined unstained under direct illumination, they may be seen to contain a great variety of materials some of which are not visible in control preparations of normal corneas. These may be partly resolved into the following groups.

1. Vaccine bodies, which are easily recognized by their relatively large size, low refractive index, and their tendency to be closely associated with the nuclei. Two of them are illustrated in Fig. 18, which

² This material was kindly placed at my disposal by Dr. Noguchi. It consisted of two rabbits 1 day after infection with vaccine prepared by a special method (Noguchi, H., Pure cultivation *in vivo* of vaccine virus free from bacteria, *J. Exp. Med.*, 1915, xxi, 539) by which it is freed from bacterial contamination, two 2 days after infection, three 3 days, two 4 days, one 5 days, three 6 days, one 7 days, one 8 days, one 9 days, and three controls, making nineteen in all.

should be compared with Fig. 1 representing a similar cell from an unvaccinated cornea.

2. Highly refractile spherical droplets which are usually gathered together in clumps and are probably of lipoidal nature.

3. A few scattered, rod-shaped mitochondria which may be definitely identified by the addition of a little Janus green, which colors them specifically when used in sufficient dilution (Fig. 20).

4. Large masses of coccal and bacillary shaped bodies of low refractive index, as represented in Fig. 19. Their occurrence is variable and does not seem to parallel the vaccine reaction. No independent motility could be established. They may be temporary phase granules owing their appearance to some alteration in osmotic conditions, or they may represent an accidental contamination with bacteria.

5. Large, highly refractile droplets of neutral fat which vary considerably in number, resist the solvent action of 1.5 per cent acetic acid drawn under the cover-glass, and are blackened by treatment with osmic acid.

Through the addition of brilliant cresyl blue 2 B³ to the physiological saline solution in a concentration of about 1:25,000, the vaccine bodies are almost instantly stained a color varying from pink to dark blue (Figs. 3 to 17) and the stages in their formation may easily be studied. Why von Prowazek (1912) failed to obtain a definite color reaction with this dye I am unable to suggest, unless the sample used was of different constitution or employed in a different way.

As a control measure brilliant cresyl blue was applied to testicle lesions with the same result. It was found that the same material will stain a brick-red color with neutral red and that Janus green will color it when sufficiently concentrated. Unfortunately, staining with these dyes is largely a process of selective adsorption, so that it does not convey any information regarding the chemical constitution of the material stained. But it is, perhaps, not without significance, regarding the nature of vaccine bodies, that the dyes are true solutions commonly used for the study of cellular constituents, and that they have achieved no popularity as an aid to the study of microorganisms.

³ For the manufacture of brilliant cresyl blue 2 B see Schultz (Schultz, G., Farbstofftabellen, Berlin, 1914).

Although they are relatively non-toxic (Hogue, 1922), these methods of supravital staining are open to serious question unless very carefully controlled. Since the cells are slowly dying, not through any toxic action of the stain but on account of traumatism and unfavorable environmental conditions, it is important not to prolong observations more than 30 minutes after the cells are removed from the cornea; certainly the moment that they show evidences of shrinkage, or of the adsorption of fluid with the release of molecular bombardment causing brownian movement, or of coloration of the nuclei, the preparations should be discarded.

The fragmentary appearance of the smaller vaccine bodies precludes any possibility of confusion with the segregation apparatus, originally described by Renaut, which is brilliantly colored by this technique in certain blood and connective tissue cells in which it takes the form of clusters of rounded droplets. Another point of distinction is that in smallpox similar bodies are found within the nuclei. A high development of this apparatus is suggested by Simpson (1922) as a criterion for the identification of monocytes.

With the fact in mind that in some respects the study of fixed tissues is less likely to be misleading because in them all vital activities are simultaneously arrested in some definite physiologic phase, every effort has been made to extend and to correlate both lines of inquiry. Many fixatives,⁴ stains,⁵ and microchemical methods⁶ have been applied to infected and normal cornea and testicles.⁷

⁴ Zenker's fluid with and without acetic acid, formalin, Regaud's fluid, Giemsa's sublimate mixture, acetic-sublimate, absolute alcohol, Altmann's fluid, Bensley's acetic-osmic-bichromate mixture, osmic acid in various concentrations, da Fano's modification of Cajal's silver method, etc., were used. In addition, "*Klatsch*" preparations, made as suggested by Ewing, were fixed by heat, by the vapor of formalin and osmium, by absolute alcohol, and in other ways.

⁵ The stains used were Borrel's, Giemsa's, Löffler's methylene blue, Mallory's eosin and methylene blue, Wright's stain, iron-hematoxylin, Pappenheim's pyronine and methyl green, carbolfuchsin-acid violet, aniline fuchsin-methyl green, Herxheimer's Scharlach R, etc.

⁶ Mayer's muchematein for mucus, the Macallum-Bensley iron reaction as suggested by Nicholson, Millon's reagent, and Schultze's oxidase reaction.

⁷ I am also indebted to Dr. Noguchi for allowing me to study his extensive series of stained preparations of vaccinia in rabbits and in calves.

In corneal cells supravitaly colored with brilliant cresyl blue, the irregularity in the form and distribution of the material from which the vaccine bodies are built is most noticeable. It varies from the very smallest aggregates, probably even beyond the limits of microscopic visibility, which do not concentrate the dye, and which cannot be resolved with the aid of the best lenses (Fig. 3), to roughly spherical masses upwards of $5\ \mu$ in diameter (Figs. 11, 12, and 15). This variability is exhibited by neighboring cells in almost any active stage of the process from 2 to about 9 days after vaccination. This is shown with equal clearness in fixed tissues (Figs. 21 to 45). After search through a number of preparations, or even in a single section, masses of material may be selected which correspond in size and shape to the elementary corpuscles, initial bodies, and reaction bodies described in the literature;⁸ but considered all in all, so haphazard is their morphology that it is difficult to satisfy oneself of the existence of a progressive series of morphological changes indicative of independent growth other than a simple increase in size of the aggregates through the accretion of more and more material. It is equally difficult to make out a breaking down of the large masses with a liberation of the original tiny fragments. Fig. 35 may represent a dispersal of elementary corpuscles, or it may represent a stage in the condensation of small bodies into a larger mass, depending upon which way it is interpreted.

That we are in fact dealing with a tremendous development of material which does not owe its ultimate origin to an infective process is shown by the demonstration of traces of a similar substance when the same technique is applied to the unvaccinated cornea. Its substantial similarity, as observed in normal cells supravitaly colored with brilliant cresyl blue and in normal cells after fixation in Zenker's fluid (less acetic acid) and staining with Giemsa's stain may be seen by comparing Figs. 2 and 22. There is also a close parallelism between its increase in amount, under the influence of vaccination, as one follows it in supravitaly stained cells and in fixed tissues. When colored with a relatively strong solution of Janus green

⁸ A close comparison may be drawn with Figs. 2 to 6 in the section on vaccine in von Prowazek's handbook to protozoa.

(1:10,000) the material changes to pink and bleaches with the formation of the color base and leuco base on reduction at the same time do the other cytoplasmic constituents. After fixation in Altmann's fluid, and other mixtures containing osmium, it is, like the chromidial substance in gland cells, somewhat more diffuse. It would be unsafe to say that it occurs in definite aggregates in normal living cells *in vivo*, for the technique of its demonstration in living cells and in fixed tissues may easily, and probably does, produce artificial changes in the colloidal substratum of the cytoplasm. In other words, the material, as we see it, is the expression of the treatment we accord the cells; its occurrence in small quantities is not dependent upon vaccination.

Owing to their larger size, the vaccine bodies lend themselves more easily to microchemical analysis than does this primordial material, but beyond the statement that they are in part, at least, of protein nature, little may be said with certainty. Their resistance to tryptic and peptic digestion has been noted by von Prowazek (1912). Attempts to secure a definitely positive reaction to Millon's reagent and to the Bensley-Macallum test for iron have been unavailing, but my failure may be due to technical difficulties. Neither do they respond to Schultze's reaction for oxidase granules. They are almost immediately dissolved when a 1.5 per cent solution of acetic acid is drawn under the cover-glass. Since vaccine bodies which are apparently similar develop in the interstitial cells of the testis, and are recorded in the literature in many other locations, it follows that the original material is to be regarded as a fundamental constituent of protoplasm.

The phagocytosis of entire leucocytes and of other corneal cells is a phenomenon of common occurrence (Figs. 16, 39, and 40), but I think that the vaccine bodies do not arise through the digestion of red blood cells,—an old idea which Woodcock (1921) has recently reaffirmed,—because I have not been able to distinguish any traces of hemoglobin coloration in freshly teased corneal cells. The test for masked iron, on which he places so much reliance, can hardly be expected to differentiate between iron-containing compounds of hematogenous and nuclear origin. Moreover, the properties of the bodies, as detailed above, and the occurrence of minute deposits of similar material in unvaccinated corneas are not easily reconciled with this view. The

theory that they are formed through the phagocytosis of leucocytic fragments breaks down in the absence of data regarding consecutive stages in the process. In the normal cornea there is little or no breaking down of leucocytes, and after vaccination the leucocytic increase is somewhat variable and does not closely parallel the development of definitive vaccine bodies. Neither are vaccine bodies more abundant, or larger, near foci of leucocytic destruction.

It seems more likely that the material of which they are composed is endogenous. That it is not related to mitochondria is evident from the application of the Janus green reaction (Fig. 20) and from the general appearance of mitochondria in fixed preparations of the cornea (Fig. 45). Although I have not been successful in obtaining satisfactory slides of reticular material (Golgi apparatus) in the cornea, silver preparations of vaccinated testicles indicate that it does not constitute a source of confusion. In consideration of Ewing's (1904-05) studies with "*Klatsch*" preparations, a nuclear origin is strongly indicated, some investigators going so far as to suggest that in studying vaccine bodies we are dealing wholly or in part with extruded nucleoli. The avidity with which the vaccine bodies take up basic dyes and their topographic association with the nuclei are worthy of comment in this connection. The failure of brilliant cresyl blue to bring to light similar material within the nuclei (Figs. 2 to 17) does not militate against this conception because the dye is not able, in dilute solution and in so short a space of time, to penetrate through the nuclear membranes. At present it seems only safe to conclude that the material may be in part of nuclear origin. It would be very difficult to prove that it is wholly so.

When, under the stimulus of vaccination, the material is deposited in fairly large masses, it shows evidences of heterogeneity in both living cells and in fixed tissues. Supravital staining with brilliant cresyl blue reveals the presence of blue-staining droplets, or granules, within the larger pink-staining masses. The blue droplets vary in size, but only in rare instances are they as large as the pink-staining material (Fig. 17). They are also of less frequent occurrence. In some cases the vaccine bodies contain masses of high refractive index and irregular shape, which do not stain and which appear to be of less fluid consistency. Occasionally these occur free in the cytoplasm.

Fixed preparations also furnish evidence of corresponding diversity in composition. Similar droplets are visible in the vaccine bodies after a variety of fixations, of which Zenker, acetic-sublimate, formalin, and Regaud may be mentioned. They remain uncolored after treatment with the Borrel combination (as suggested by Calkins), Mallory's eosin-methylene blue, carbolfuchsin-acid violet, and other dyes. The chromatophobe, refractile masses, above mentioned, have not been seen after fixation. With Wright's stain after Zenker fixation, a rough gradation may be established between small irregular vaccine bodies which are colored dark blue and certain larger ones in shades of gray which take a pink or a light robin's egg blue color, as represented in Figs. 27 and 36. Sometimes these larger masses are homogeneous, or they may be surrounded with a fringe of dark blue material, as indicated in Fig. 36. Staining with aniline methyl violet, treating with Gram's iodine solution, and differentiating in 95 per cent alcohol discloses a similar heterogeneity, as shown in Fig. 40. Giemsa's stain (Fig. 41) brings to light much the same effect, it being possible to modify at will the color values from dark blue to reddish blue by inserting traces of colophonium in the alcohol of differentiation, as suggested by Wolbach, Todd, and Palfrey (1922) for the demonstration of *Rickettsia*.

From these observations I infer that the material is labile, and that, as the larger vaccine bodies are formed, it undergoes some dissociation or takes up substances from the cytoplasm so that it loses its original optical homogeneity; but this change can hardly be regarded as suggestive of the presence in it of independent microorganisms in the hypothetical stage of initial bodies.

Neither is the physical behavior of the material suggestive of the presence in it of microorganisms. In cells which are still living, it has not been possible to detect evidence of independent motility. The smallest bodies show a tendency to be distributed fairly uniformly throughout the cytoplasm and the larger ones to be congregated near the nuclear membrane. In common with other cytoplasmic materials its form and position are determined by cellular stresses, by electrical charge, and by other physical and chemical forces. For example, in the depth of the lesion, where the cells are often pressed together laterally, the deposits of material fre-

quently assume an elongated shape and become disposed parallel to the long axis of the cell. When the shape of the cell is altered through manipulation, the shape assumed by the material also changes and in some cases it seems to flow upon the surface of the nucleus, as is shown in Figs. 5 and 9, but the movement exhibited is in no sense independent or ameboid. That the material is very fluid in consistency and exhibits but little internal organization requires no further proof. The larger masses are perhaps more fluid than the smaller, more angular fragments, and have rounded up into spheres by virtue of the law of least surfaces.

In this respect also the study of permanent preparations is confirmatory. The habit the material shows of conforming in shape to that of the cell under pressure is illustrated in Fig. 28—its flowing motion in Fig. 30. As in the living cells, so also here the larger masses are spherical, but they show another property in their tendency to lodge in infoldings of the nuclear membrane. Sometimes they appear to press into the nucleus from opposite sides (Fig. 33), while in other cases they are found in deep invaginations of its surface, which may be single or multiple, and which convey the impression, when cut in transverse section, that the vaccine bodies are actually intranuclear, as represented in Figs. 34, 38, and 40. But pictures of this kind are not so easily interpreted. Whether the vaccine bodies actively indent the nuclei by virtue of being of more solid consistency, or whether they flow into depressions produced in the yielding surfaces of the nuclei through shrinkage, it is difficult to say. It seems clear, however, that the indentations are artificial since they are not to be seen in living cells (compare Figs. 3 to 20).

Considerable attention has been devoted in the literature to the areas of clear cytoplasm, or halos, which surround the vaccine bodies (Figs. 23 to 35). Investigators have called to mind approximately similar halos as accompaniments of bacteria in tissues. The fact that they are not visible in the case of vaccine bodies supravitaly stained with brilliant cresyl blue is not very helpful, because neither do I find the bacterial halos in insect material. Nevertheless, it can hardly be denied that their appearance is strongly suggestive of shrinkage at interfaces between fluids of different consistency and composition. That their extent is in some measure proportional to the size of the

vaccine bodies will be seen by comparing Figs. 23 and 27 with Fig. 33. The presence of slight halos about the nuclei (Figs. 25 and 26), though probably produced in the same way would hardly be taken to mean that the nuclei behave like independent microorganisms.

In discussing the significance of cell inclusions in scarlet fever, Mallory (1903-04) expressed the opinion that:

"In order to prove that a series of bodies are stages in the developmental cycle of a protozoon we are dependent on three things, namely, ameboid motion, definite and characteristic morphology of the various bodies found, and the demonstration that the bodies go through a progressive series of changes or developmental cycle resulting in increase in size followed by division and the re-formation of the small bodies from which the series started."

From the foregoing account it is apparent that I have not been able to fulfill any of these conditions.

The problem is complicated by the fact that well formed vaccine bodies have been repeatedly recorded outside the cells. In some of my preparations certain bodies, bearing a resemblance, perhaps entirely superficial, to intracellular vaccine bodies, are to be seen in the fibrous tissue beneath the epithelial layer of the cornea. Whether they have been formed *in loco* or have escaped from degenerating epithelial cells or leucocytes cannot be definitely stated in the absence of specific criteria for their identification. The chlamydozoal hypothesis does not provide for their development apart from the cells. Attention has already been directed to the fact that according to this theory they are said to be of dual nature, consisting of intracellular parasites in the form of initial bodies cloaked in a mantle of material elaborated by the cell. This assertion belongs to the category of statements which are easy to make and exceedingly difficult either to prove or to disprove. If the granules described by MacCallum and Oppenheimer are in fact the infective agents, and, if they maintain throughout their life cycle the morphological and tinctorial properties ascribed to them, then it appears to be unlikely that the virus is contained within the vaccine bodies. But, should the virus be beyond the limits of visibility with the best lenses, then its relation to the vaccine bodies is not easily determined.

While I have not made a cytological study of other diseases attributed to chlamydozoal organisms, a careful review of the literature does indicate, that, as far as the nature of the visible cytoplasmic granulations is concerned, their inclusion in a single category is scarcely warranted. Particularly does this appear to be the case in trachoma (Solovief, 1921).

In comparing the properties of the granules described by MacCallum and Oppenheimer in vaccine lymph with those of typical intracellular vaccine bodies, several points of similarity and of dissimilarity may be noted. (a) Like the granules of vaccine lymph the vaccine bodies are not specifically colored with trypan blue, and are colored various shades of blue and red with Wright's stain. They apparently surpass the granules of vaccine lymph in the intensity of their coloration with Löffler's methylene blue and especially with carbolfuchsin. (b) Unlike them, they are Gram-positive in the sense that they resist decoloration with alcohol after staining with aniline methyl violet and treatment with Gram's iodine. They are, moreover, intensely stained with neutral red. But still more striking is the difference in morphology. Instead of being characterized by their uniformity, the vaccine bodies are extremely variable. They bear no resemblance to tiny beaded chains of streptococci, as do the granules in vaccine lymph described by MacCallum and Oppenheimer.

To the claim that these reactions are not specific and mean but little one way or the other,—which is freely admitted,—and, further, that one would not expect the granules in vaccine lymph to maintain their original uniformity of morphology after penetration into the cell where they find themselves under entirely different surroundings, it is but natural to advance the observation of the existence of traces of material similar to the vaccine bodies in unvaccinated corneal cells. If the granules which these investigators have described are in truth the infective agents, they cannot be taken to correspond with material seen in uninfected cells, however imperfect our knowledge of its chemical constitution may be.

Since, however, the report of MacCallum and Oppenheimer is of a preliminary nature, many important details being reserved for a subsequent contribution, it seemed desirable to obtain lymph from the

same source and to repeat their work in order to make a more direct comparison with intracellular vaccine bodies.⁹

Bearing in mind the results obtained by MacCallum and Oppenheimer, I took about 5 cc. of this lymph, centrifuged it at high speed for 1 hour, drew off the turbid supernatant fluid,—which in their experiments contained the virus,—and divided it into six portions. Through the addition of varying amounts of glycerol to samples of Locke's solution, solutions were made up to the following specific gravities, determined by hydrometer: 1.11, 1.12, 1.13, 1.14, 1.15, and 1.16. One of these fluids was added to each of the six portions and centrifuged. Although the fresh supernatant fluid was examined in each case, no aggregates suggestive of organisms were found. In attempting to prepare permanent specimens, the relatively large amount of glycerol constituted a considerable handicap. Fixation in formalin vapor and absolute alcohol proved unsuccessful, but after prolonged treatment of smears with the vapor of a slightly warmed 2 per cent solution of osmic acid followed by desiccation, it was possible to color certain granulations intensely blue with Giemsa's stain in the lymph which had been centrifuged in a fluid of 1.14 specific gravity. It will be recalled that MacCallum and Oppenheimer believe the specific gravity of the virus to be about 1.12 or 1.13.

On close examination these granules were seen to be roughly spherical and to vary in diameter from 0.2 to 0.4 μ . Sometimes the larger ones are slightly angular. They occur free or embedded in a loose coagulum which stains a light lilac color. Although they are quite frequently grouped end to end in series, the individual segments are not always of the same size and rarely exceed four or five in number. The same granules are tinted gray, not black, with osmic acid. Judging from the brief description by MacCallum and Oppenheimer their appearance is only remotely suggestive of the more uniform bodies which they found.

⁹ I am again indebted to Dr. Noguchi for his kindness in giving me a large supply of vaccine lymph which he obtained from the H. K. Mulford Laboratories through the courtesy of Dr. W. F. Elgin.

CONCLUSIONS.

Vaccine bodies in living corneal cells may be specifically stained by the addition of a small quantity of brilliant cresyl blue 2 B to the physiological salt solution in which they are being observed. Their appearance by this method (Figs. 3 to 17) corresponds with that seen in fixed preparations (Figs. 22 to 42). Both lines of study reveal the existence of traces of similar material in unvaccinated corneal cells. As this increases in amount during the reaction, it behaves like an integral, cytoplasmic constituent of fluid consistency and shows no evidence of being endowed with any measure of independent vitality. The low grade of structural differentiation which it does exhibit, in living cells as well as in fixed tissues, is not suggestive of the presence within it of independent microorganisms. The material differs radically in its morphology and microchemical reactions from the granules observed by MacCallum and Oppenheimer in vaccine lymph.

BIBLIOGRAPHY.

- Calkins, G. N., The life history of *Cytoryctes variolæ*, Guarnieri, *J. Med. Research*, 1904, xi, 136.
- Councilman, W. T., Magrath, G. B., and Brinckerhoff, W. R., I. The pathological anatomy and histology of variola. *J. Med. Research*, 1904, xi, 12.
- Ewing, J., The structure of vaccine bodies in isolated cells, *J. Med. Research*, 1904-05, xiii, 233.
- Gins, H. A., Untersuchungen über die für Variola und Vaccine spezifischen Zellveränderungen, *Z. Hyg. u. Infektionskrankh.*, 1922, xcv, 255.
- Harde, E. S., Some observations on the virus of vaccinia, in Mémoires publiés à l'occasion du Jubilé de Élie Metchnikoff (16 Mai 1915), Paris, 1921, xii, 107.
- Hogue, M. J., A comparison of an amœba, *Vahlkampfia patuxent*, with tissue culture cells, *J. Exp. Zool.*, 1922, xxxv, 1.
- Jungmann, P., Das wolhynische Fieber, Berlin, 1919.
- Lipschütz, B., Die ätiologische Erforschung der "Einschlusskrankheiten" der Haut, *Zentr. Haut- u. Geschlechtskrankh.*, 1921-22, iii, 3.
- MacCallum, W. G., and Oppenheimer, E. H., Differential centrifugalization: A method for the study of filterable viruses, as applied to vaccinia, *J. Am. Med. Assn.*, 1922, lxxviii, 410.
- Mallory, F. B., Scarlet fever. Protozoon-like bodies found in four cases, *J. Med. Research*, 1903-04, x, 483.
- Paschen, E., Über den Erreger der Variolavaccine. Immunitätsverhältnisse bei Variolavaccine, in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1911, suppl. 1, 465.

- von Prowazek, S., Chlamydozoa. I. Zusammenfassende Übersicht, *Arch. Protistenk.*, 1907, x, 336.
- von Prowazek, S., Vaccine, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 122.
- von Prowazek, S., and Lipschütz, B., Chlamydozoen. Allgemeines, in von Prowazek, S., Handbuch der pathogenen Protozoen, Leipsic, 1912, i, 119.
- da Rocha-Lima, H., Beobachtungen bei Flecktyphusläusen, *Arch. Schiffs- u. Tropen-Hyg.*, 1916, xx, 17.
- Ségal, J., The association of the virus of typhus fever with the various blood elements, *Brit. J. Exp. Path.*, 1922, iii, 95.
- Simpson, M. E., Vital staining of human and mammalian blood, with special reference to the separation of the monocytes, *Anal. Rec.*, 1922, xxiii, 37.
- Solovief, P., Examen histologique de quelques pièces de trachome expérimental du lapin et du magot, *Arch. Inst. Pasteur*, 1921, i, 388.
- Steinhardt, E., Israeli, C., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia, *J. Infect. Dis.*, 1913, xiii, 294.
- Steinhardt, E., and Lambert, R. A., Studies on the cultivation of the virus of vaccinia. II, *J. Infect. Dis.*, 1914, xiv, 87.
- Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922.
- Woodcock, H. M., An introduction to the study of hæmatophagy, *J. Roy. Army Med. Corps*, 1921, xxxvii, 418.

EXPLANATION OF PLATES.

PLATE 58.

The figures have been drawn with a Zeiss apochromatic objective of 1.5 mm., compensating ocular 6, and camera lucida at the table level, giving a magnification of 1,500 diameters. All represent living corneal cells in physiological saline solution viewed by direct illumination. Figs. 1, 18, and 19 are unstained cells. Fig. 20 is a cell supravitality stained with Janus green B and the remainder are of cells stained with brilliant cresyl blue 2 B. The corpuscles of Guarnieri, or vaccine bodies, were colored pink and blue, while the nuclei remained quite unstained.

FIG. 1. Living, unvaccinated, and unstained corneal cell in which rod-like mitochondria and other granular material may be distinguished.

FIG. 2. Cell from the same animal, supravitality stained with brilliant cresyl blue, showing traces of the material of which the vaccine bodies are built.

FIG. 3. Cell 4 days after vaccination, supravitality stained in the same way, showing a larger amount of the same material.

FIG. 4. Cell 5 days after vaccination similarly treated. The material shows signs of heterogeneity in the form of clear unstained vacuoles.

FIG. 5. Cell 4 days after vaccination, similarly stained, showing the spicula along the surface of the nucleus of a vaccine body, in which it is possible to distinguish materials of different staining reaction.

FIG. 6. Cell from the same cornea, stained in the same way, containing a larger vaccine body, exhibiting both unstained vacuoles and blue-stained granules.

FIG. 7. Cell 2 days after vaccination similarly stained.

FIG. 8. Cell 5 days after vaccination, stained in the same way and containing a heavy deposit of material.

FIG. 9. Cell 2 days after vaccination stained in the same way.

FIG. 10. Cell 1 day after vaccination, containing several small heterogeneous vaccine bodies.

FIG. 11. Cell 5 days after vaccination, with a very heavy deposit of material.

FIG. 12. Cell 5 days after vaccination, with a large solitary vaccine body.

FIG. 13. Cell 2 days after vaccination, containing a solitary vaccine body in which large and small droplets may be distinguished, which took the blue stain.

FIG. 14. Cell 5 days after vaccination, containing several vaccine bodies with droplets (or granules) which took a blue coloration.

FIG. 15. Cell 5 days after vaccination, containing a very large solitary and apparently homogeneous vaccine body.

FIG. 16. One corneal cell phagocytosed by another in a 5 day lesion.

FIG. 17. Cell from the same cornea, containing a vaccine body in two parts staining differently.

FIG. 18. Unstained corneal cell 7 days after vaccination, containing two large and slightly refractile vaccine bodies closely applied to the nuclear membrane. The finer granulations may be resolved into indistinct rod-like mitochondria and a few highly refractile droplets of lipoid-like material.

FIG. 19. Corneal cell from the same lesion, also unstained. It contains a mass of coccid and bacillary bodies which is only very occasionally met with.

FIG. 20. Cell from a 7 day vaccination, supravitally stained with Janus green to demonstrate more sharply the mitochondria. It contains a vaccine body as large as the clear unstained nucleus.

PLATE 59.

These figures have been drawn under the same magnification. All of them represent fixed and stained preparations. At least two factors are concerned in their small size, as compared with Figs. 1 to 20: first, the shrinkage resulting from the technique employed, and second, the tendency of the living cells illustrated in Figs. 1 to 20 to become slightly flattened, thus increasing their apparent size, but not their actual volume.

FIG. 21. Normal corneal cell from a control animal fixed in Zenker's fluid and colored with Giemsa's stain. The irregular deposits of material in the cytoplasm are colored dark blue in the original preparation. On infection this gives rise to typical vaccine bodies.

FIG. 22. Cell about 2 mm. from the lesion in a cornea 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain. The same irregular material is again colored dark blue.

FIGS. 23 and 24. Cells 5 days after vaccination, fixed and stained in the same way, showing an increase in the material giving rise to vaccine bodies.

FIG. 25. Cell 6 days after vaccination, fixed and stained in the same way, representing very high development of the small bodies.

FIG. 26. Cell 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, illustrating the formation of larger vaccine bodies.

FIG. 27. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg color), partly surrounded by a mantle of dark blue material, and other vaccine bodies disposed just beneath the cell membrane.

FIG. 28. Cell 4 days after vaccination, fixed in Zenker's fluid and stained with carbolfuchsin and acid violet which shows up the vaccine bodies in crimson against a light green background. It indicates an accommodation in the shape and position of the vaccine bodies to that of the cell.

FIG. 29. Cell from a 4 day lesion, fixed in acetic-sublimate and colored with Giemsa's stain, containing a very deeply colored and homogeneous nucleus.

FIG. 30. Cell from a 5 day lesion, fixed in Zenker's fluid and colored with Giemsa's stain, showing a large vaccine body containing clear droplets of uncolored material, closely applied to the nuclear membrane.

FIG. 31. Cell 1 day after vaccination, fixed in Zenker's fluid without acetic acid and colored with Giemsa's stain, containing a solitary spherical vaccine body.

FIG. 32. Cell from the same preparation possessing a smaller single vaccine body.

FIG. 33. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing the hour-glass-like indentation of the nucleus by the vaccine bodies.

FIG. 34. Cell 6 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, showing vaccine bodies occupying depressions in the nuclear membrane.

FIG. 35. Cell 2 days after vaccination, fixed in Giemsa's sublimate mixture and colored with Giemsa's stain, showing solitary and accessory vaccine bodies.

FIG. 36. Cell from a lesion of 6 days, fixed in Zenker's fluid and colored with Wright's stain, containing a large vaccine body stained light blue (robin's egg tint), with an irregular envelope of very dark purplish blue material.

FIG. 37. Cell 5 days after vaccination, representing the pocket-like indentation of the nucleus.

FIG. 38. Cell from the same preparation as Fig. 34.

FIG. 39. One corneal cell phagocytosed by another containing well developed vaccine bodies, 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain.

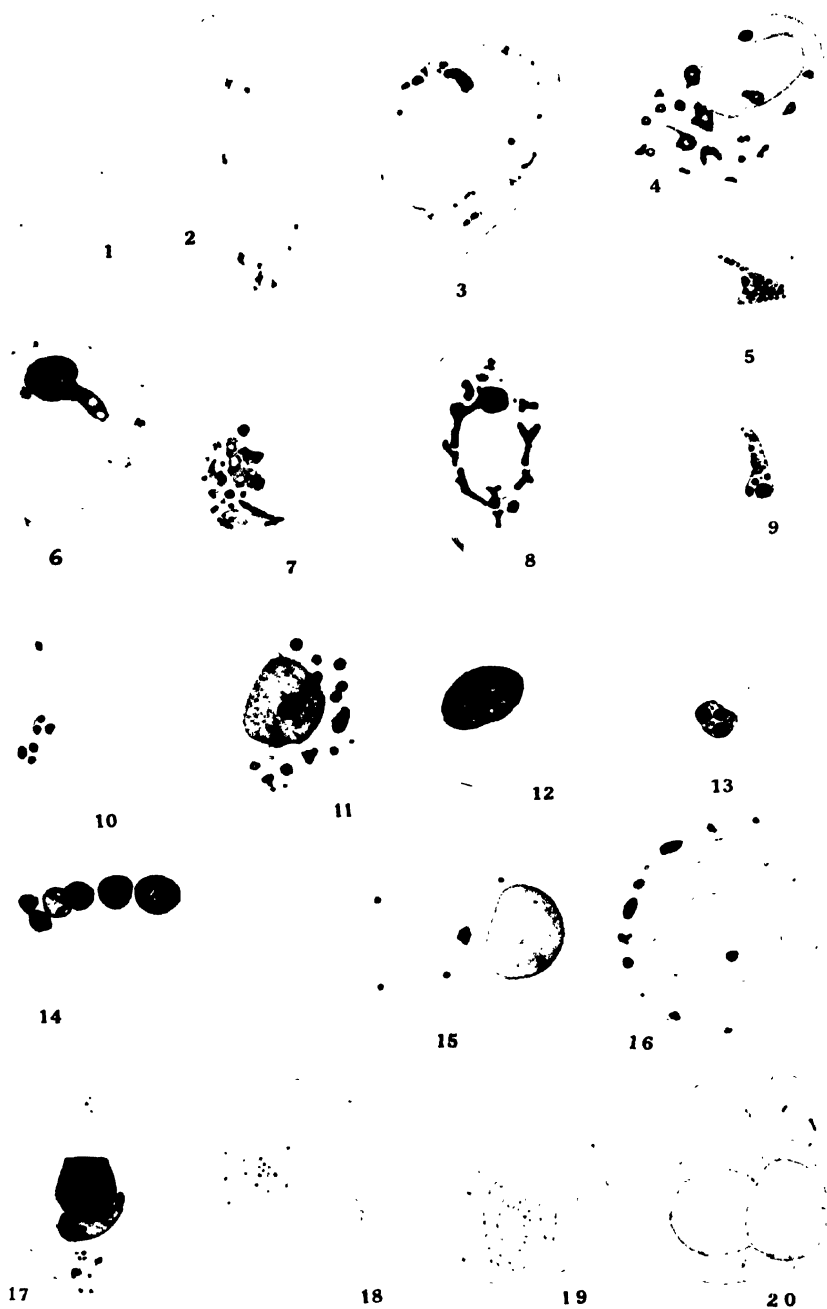
FIG. 40. A similar act in phagocytosis, in which both cells contain vaccine bodies, from a cornea 6 days after vaccination, fixed in Zenker's fluid, stained with methyl violet, treated with Gram's solution, and decolorized in 95 per cent alcohol. The vaccine bodies retain the stain much more strongly than do the nuclei. One of the bodies in the phagocytosed cell is made up of both Gram-positive and Gram-negative material.

FIG. 41. Cell from a cornea 5 days after vaccination, fixed in Zenker's fluid and colored with Giemsa's stain, containing a large vaccine body, the center of which was colored dark blue and the periphery light blue.

FIG. 42. Cell from the same preparation as Fig. 35, illustrating also nuclear infoldings and halos.

FIGS. 43 and 44. Constricted and binucleated cells from the same specimen as Fig. 41.

FIG. 45. Cell distant about 4 mm from the lesion in a cornea 3 days after vaccination, fixed in Altmann's fluid and stained with fuchsin and methyl green. The mitochondria are rod-like and filamentous. They stained bright red in sharp contrast to the homogeneous green-colored cytoplasm in which the material destined to form the vaccine bodies is not visible.



(Cowdry: Supravital staining of vaccine bodies.)



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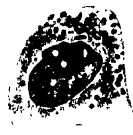
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(Cowdry: Supravital staining of vaccine bodies.)

A METHOD FOR THE PERMANENT STERILE DRAINAGE OF INTRAABDOMINAL DUCTS, AS APPLIED TO THE COMMON DUCT.

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PLATE 4.

(Received for publication, July 28, 1922.)

There have been many attempts to maintain rubber tubes in connection with the common ducts of animals for the purpose of bile collection over considerable periods of time. They have been so uniformly unsuccessful as to warrant the axiom that such tubes will come away within a few days. This has regularly proved the case in our experience until recently when, happening to leave within the peritoneal cavity a longer portion of the tube than usual, we found it firmly fixed in place although nearly 2 weeks had elapsed. No sign was present of ascending infection along the rubber with destruction of the duct wall next the cannula, which had previously terminated the observations. Instead, the tube was thinly but closely sheathed in omentum which met and joined the common duct, so that the entire collecting apparatus was covered. Elsewhere throughout the peritoneal cavity there were no adhesions. Acting on the suggestion thus presented, we have developed a method whereby the total bile may be collected in a sterile state day after day, during months certainly and probably during years. The method will be described at some length, since it obviously has a wider application than that for which it was devised.

The need for an exact means of bile collection is sufficiently indicated by the fact that major problems in connection with the bile output which were first formulated nearly three generations ago are at this day still unsettled.

Schwann¹ devised in 1841 the fistula method that is still employed in routine studies of the bile. With its aid much has been learnt of the relation of the secretion to body processes. The operation required is nowadays simple, mere severance and ligation of the common duct and cholecystostomy through a separate wound; but the after-care of the animals and the attention required if one is to obtain useful specimens have become no less in the 70 years since Bidder and Schmidt² published their classical observations on fistula bile. Furthermore there are natural limitations to the method which can scarcely be overcome. 24 hour specimens are difficult to obtain unless the animal is suspended in a sling. Dastre,³ it is true, succeeded in keeping a tube in the fistula wound for more than a year in two dogs ("*Cholin*" and "*Cholite*"), collecting the bile from day to day into a bag suspended at the neck; but few have attempted to repeat his feat. The fistulous opening in the abdominal wall is often pressed shut by contraction of the abdominal muscles, or that of scar tissue, with a resulting intermittent obstruction and not infrequently reopening of the passage to the intestine, as Schwann himself noted. Usually there is a cholangitis,⁴ and a more or less copious secretion from the ducts mingles with the bile; while if the gall bladder remain normal it cannot but exert a marked influence on the fluid coursing through it.⁵

Of the less used methods of bile collection, that devised by Pawlow⁶ has given highly significant results. It was designed primarily to show the relation of feeding to the delivery of bile into the intestine, and has most value in this connection. A flap of duodenal wall containing the ampulla of Vater is reflected, sewn fast to the skin surface, and the opening in the duodenum is closed. The bile flow may then be directly observed. Pearce and Eisenbrey⁷ have suggested that the bile be diverted into the urine by anastomosis of the common duct with a ureter. And recently Mann⁸ has connected the common duct directly with the body surface by a two stage operation. He reports that the fistula is less likely to close than that of a cholecystostomy.

Method.

The success of our method lies, as already indicated, in the interpolation, with special care for asepsis, of a long drainage tube between

¹ Schwann, T., *Arch. Anat. u. Physiol.*, 1844, No. 2, 127.

² Bidder, F., and Schmidt, C., *Die Verdauungssäfte und der Stoffwechsel*, Milan and Leipsic, 1852.

³ Dastre, A., *Arch. physiol.*, 1890, ii, series 5, 714, 800.

⁴ Pelkan, K. F., and Whipple, G. H., *J. Biol. Chem.*, 1922, 1, 513.

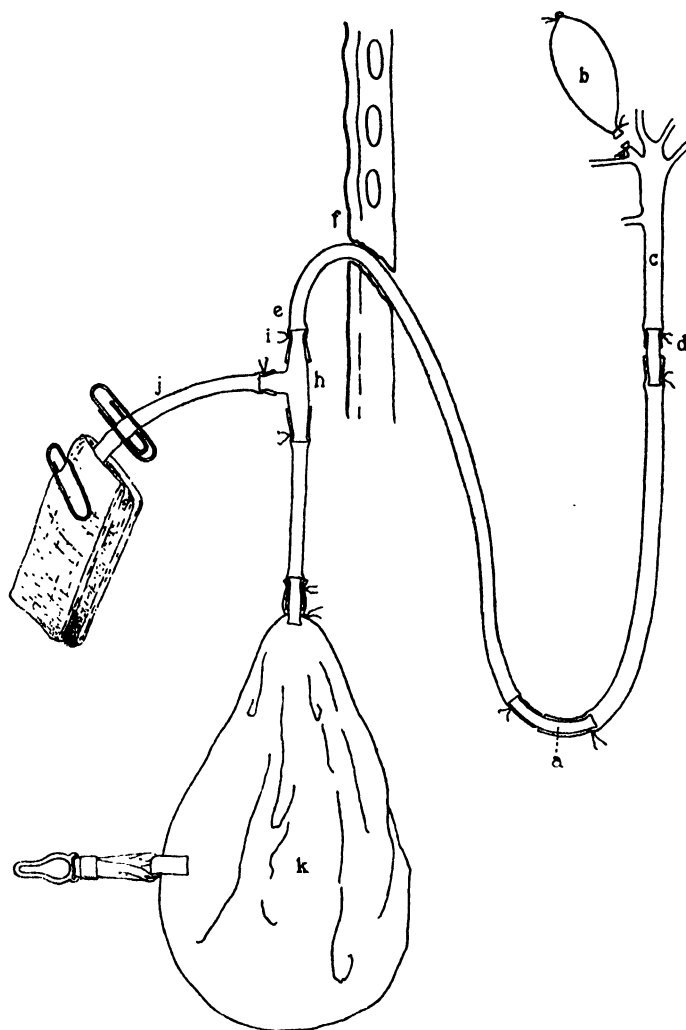
⁵ Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

⁶ Pawlow, J. P., *Arch. Physiol.*, 1887, 452, cited by Langendorff, O., *Ergebn. Physiol.*, 1902, i, pt. 2, 272.

⁷ Pearce, R. M., and Eisenbrey, A. B., *Am. J. Physiol.*, 1913, xxxii, 417.

⁸ Mann, F. C., *J. Lab. and Clin. Med.*, 1921-22, vii, 84.

the common duct and the opening in the abdominal wall. The tube must be pliable near the cannula, else this latter may be pushed askew



TEXT-FIG. 1.

through leverage exerted by the viscera, with obstruction as result; and it must be resistant where it passes the abdominal wall to sustain muscular contraction. To gain these ends a curved glass tube

(Text-fig. 1, *a*) is employed with, on one limb, the soft black rubber tubing that connects with the cannula, and, on the other, a piece of red duodenal tubing that is to pass through the abdominal wall. The entire U of rubber and glass should be as long as the abdominal cavity will readily permit—10 to 12 cm. in a large animal.

With a view to the removal of irritating substances from the surface of the rubber, we have boiled it in a weak solution of sodium bicarbonate, soaked it in weak hydrochloric acid, and washed it well in water prior to autoclaving twice. The cannula (*d*) connecting with the duct (*c*) should be as large as the latter will admit, and blunt ended, with but a slight constriction on the shank.

The skin of the dog is swabbed with iodine followed by alcohol, and, under ether, the common duct and gall bladder are exposed by a right rectus incision. The duct is tied off at once near the intestine to minimize the possibility of infection from this source during the later manipulations; the neck of the gall bladder (*b*) is severed between ligatures placed as far down as possible so as to exclude all bladder mucosa from the duct tract, and the contents of the organ is removed by aspiration, with closure of the needle hole by a purse-string suture. All is now ready for connection of the collecting apparatus. A ligature is laid, but not tied, about the duct just above where it is to be opened. With the aid of long forceps the U-tube with cannula attached is lifted out of the flask of water in which it has been sterilized. In so doing, the black rubber tube should be firmly seized just back of the cannula to close its lumen, else the water within will run out and be replaced by air of dubious sterility. As the tube comes from the flask, its other end is closed with a bulldog clamp; and the whole is at once enveloped in moist, twice sterilized gauze. The duct is cut; the cannula introduced into it without touching the surrounding structures; and the ligature already in place is tied down and supplemented with another. Medium silk is better for the purpose than fine, as having less tendency to cut through. The common duct should now be severed below the cannula and the lower end pulled away from this latter to lessen the danger of a reconstitution of the original channel to the intestine, should obstruction accidentally occur.

To bring the tube out through the abdominal wall, a small stab wound (*f*) is made with a cataract knife obliquely through the tissues just below the rib margin in the mammary line; a sharp-nosed artery forceps is thrust through into the abdominal cavity and the end of the tubing pulled forth. The stab wound should be small enough to hug the tubing closely. The U is now adjusted so that the bend lies well down toward the pelvic basin; the laparotomy opening is closed in three layers; and a dressing wet in 80 per cent alcohol is applied. Over the stab wound there should be only a small dry sponge split to encircle the tubing and dusted with powdered boracic acid.

The free end of the drainage tube is now connected with a rubber bag. This bag (*k*), a rubber balloon of 150 to 300 cc. capacity in the unstretched state, has

been subjected to the same sterilization as the U-tube. During the process it is likely to burst unless kept deflated by a wrapping of gauze. To it there has previously been joined a short length of tubing with a small glass T at the end (*h*), the stem of which bears another piece of tubing (*j*), the eventual outlet tube. The same care is taken to prevent the entrance of air on removal of the apparatus from the sterilization flask as in the case of the drainage tube. Clamps are now placed on the tubing close to the T and this latter is filled with 5 per cent phenol solution. So also is the free end of the drainage tube (*i*) which is then slipped over the open limb of the T and tied on. Prior to removal of the clamp from the drainage tube, the bag should be emptied through the outlet tube of any water it may contain. Afterward the end of the outlet is plugged with cotton wet with phenol solution and covered with a sponge similarly moistened. Ordinary paper clips of wire hold the sponge in place and press the tube shut.

Care is required in the adjustment of the dressings during the first few days to prevent obstruction from a twist or elbow in the tubing. The sponge where this latter emerges from the skin should be changed daily throughout the life of the animal. Given such care the opening will usually remain practically dry even for months, without loosening around the tube or any formation of granulation tissue (Figs. 1 and 2); but lacking it, infection soon ensues, with gaping of the opening and a seepage of thin pus. The laparotomy wound usually heals by first intention. Nevertheless a binder of muslin should be kept on permanently, to which the T-tube is moored with adhesive, as is also a rattan basket to hold the collecting balloon. This basket, oval and shallow, should be large enough to contain all of the drainage apparatus and should be fixed against the side of the animal over the stab wound. The high situation of this latter makes possible the use of male as well as female animals. The end of the outlet tube is brought out between the basket edge and the skin. A many-tailed bandage is put over all, and a snug canvas binder. A saddle of adhesive plaster over the hair of the back prevents slipping of the binders and provides fixed points from which the basket is swung.

Once in each 24 hours the binders are removed, the balloon emptied, the outlet tube plugged again, wrapped in gauze, and the abdominal opening dressed. While the emptying goes on, a clamp should be placed on the drainage tube between *e* and *f* to fend pressure from the duct system when the balloon is squeezed. The whole operation requires only a few minutes.

Lively animals by repeatedly rolling over at a time when the balloon contains bile will sometimes cause it to twist within the basket and thus produce a temporary obstruction. For this reason, balloons of flattened form are preferable to the round. They may be held from turning by a garter clasp attached to one side with adhesive and slipped over a button on the basket rim.

RESULTS.

Thus far we have collected bile by the method from seventeen dogs during periods ranging up to 3 months. When kept on a mixed diet of bread and cooked meat, the animals remain in robust health (Figs. 3 and 4), save for the development of a mild anemia later to be reported upon. In only one case has there been ascending infection along the tube with release of the cannula from the duct; and in this instance the animal had got its bandages off some days previously and pulled much of the tube out, opening it and infecting the bile. Twice the cannula slipped out of the duct end and was displaced 1 to 2 cm., because of loosened ligatures, but the tube of omentum was already so well developed when this occurred that the drainage of bile continued unaffected for some weeks, when cicatricial contraction occluded the short channel in the tissues and caused total obstruction. The general failure of infection to spread upward from the skin surface is the more remarkable because in several of our early experiments the laparotomy and stab wounds became markedly purulent. Sometimes a broad washer of rubber had been placed around the drainage tube just within the peritoneal cavity, to fix it in place, and this washer was at autopsy always found surrounded by pus and encased in massive adhesions. Yet the omental sheath further up along the rubber tube was regularly thin and uninflamed, with a smooth and glistening inner surface. Most interesting was the nature of the connection developed between duct and cannula after the collecting apparatus had been in place for some weeks. It was no longer a union by ligatures but by a thin tube of scar tissue joining with the duct and closely embracing the cannula. Only when it was cut through were the ligatures found, lying free and loose next the glass. So closely did the scar tissue hug this latter that even the pressure from a total biliary obstruction produced by clamping the drainage tube caused no leak or extension of bile along the cannula until several days at least had elapsed. Then in some instances bile penetrated between the tube and omental sheath with an eventual rupture of the latter and bile peritonitis.

Cultures of the bile were made daily in bouillon and on agar plates; and the sediment of centrifugated specimens that appeared sterile

was at intervals stained and searched. Infection of the fluid occurred sooner or later in ten of the seventeen dogs, though always with pure cultures of organisms that behaved as harmless saprophytes. In three of the instances mentioned, the infection resulted from damage to the collecting system by the animal. The circumstances under which it developed in most of the others are suggestive of infection through the blood stream. Once it was terminal in a dog that yielded sterile bile for 3 months before dying of intestinal obstruction; once it appeared in association with the prostration of a severe bronchitis; in another case it developed immediately after hard exercise on a treadmill; and twice it came early in association with a purulent laparotomy wound. We incline to the belief that in the remaining two instances careless handling of the outlet tube was responsible for the contamination. Certainly most dogs in good condition and with well healed abdominal wounds will be found to yield week in and week out a bile that is sterile.

With drainage of the bile carried on as described, there is no danger of a reopening of the pathway to the intestine unless obstruction occurs. And in our experience it has not happened even then. In instances without obstruction, the bile passages remain normal and narrow; while in obstructed ones there are the findings characteristic of stasis without infection by pathogenic organisms. Two causes of eventual obstruction have been encountered—the cicatricial contraction already mentioned that follows upon slipping of the cannula, which interfered twice; and calculus formation in the cannula, which thrice led after some weeks to a complete biliary stasis. In the other twelve instances bile secretion and collection continued over long periods until terminated by accident, or intercurrent disease, or the investigative needs.

Obstruction to the neck of the normal gall bladder is known to be followed by a gradual lessening in size of the organ which after a time contains merely a little pale, semisolid mucus.⁹ This was the regular finding in our animals.

The amount of bile present in the tube system and not evacuated when the balloon is emptied—between 2 and 3 cc. in our experiments—had, of course, to be taken into account in the quantitative work.

⁹ Mignot, cited by Aschoff and Bacmeister, who confirm the observation (Aschoff, L., and Bacmeister, A., *Die Cholelithiasis*, Jena, 1909).

DISCUSSION.

The original bile fistula operation of Schwann¹ was devised by him to determine whether bile is essential to life. And the results of the operation led him to conclude that this was the case. Later observations with animals fed a fat-free diet have made the point debatable. Dastre's³ animals were still vigorous at the end of a year. But, as Hooper and Whipple¹⁰ well point out with regard to similar findings in human beings with bile fistulas, there is no certainty that some bile did not reach the intestine, since autopsy findings are not available. These latter authors hold that the presence of a very little bile in the intestinal tract of dogs suffices for good health; but that total absence of it is not withstood, unless a diet which includes liver is fed, and even then the animals become sparsely nourished. Our first dogs did indeed lose weight rapidly, and this despite liver feeding. But in proportion as the method of drainage has been perfected, so has the condition of the animals bettered; and it is now certain that dogs on a mixed diet that is poor in fat and includes no liver remain vigorous and keep their weight during many weeks, though totally deprived of bile (Figs. 3 and 4). Practically always, though, as already stated, a moderate anemia develops early, with gradual and imperfect recovery later on. The cause of the blood changes will be the subject of a later communication.

SUMMARY.

The sheath of omentum that forms about a long rubber tube left within the peritoneal cavity provides so efficient a barrier to ascending infection that such a tube can be employed for the permanent drainage of the common duct, whereas a short tube will regularly come away after a few days. A method is described whereby the total bile of a considerable number of dogs has been obtained in a sterile state during periods up to 3 months. Save for some anemia, the animals remained in good health throughout. The method has obvious uses in connection with the collection of other secretions.

¹⁰ Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

EXPLANATION OF PLATE 4.

FIG. 1. The exit point of a drainage tube 86 days after intubation, to show the absence of reaction. The white material on and about the tube is powdered boracic acid.

FIG. 2. The characteristic sheath enclosing the portion of the drainage tube within the peritoneal cavity. The picture shows the point at which the tube passes through the parietal peritoneum. From an animal killed 25 days after the intubation.

FIGS. 3 and 4. Dogs with the collecting apparatus in place. The photographs, taken 40 days after operation, show the good general condition of the animals, and the freedom of motion permitted by the apparatus.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

(Rous and McMaster: Sterile drainage of intraabdominal ducts.)

THE INTESTINAL FLORA IN MOUSE TYPHOID INFECTION.

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(Received for publication, July 21, 1922.)

During a study of mouse typhoid caused by a strain of *Bacillus pestis caviæ*, it was found that in the course of epizootics¹ as well as among any series of mice receiving the bacilli artificially by mouth² a certain number proved refractory to infection. The mere presence of the organism, then, is not sufficient in every case to induce mouse typhoid; other factors, properly related to the specific agent, are necessary. Consequently, as the first step in an analysis of these factors, we studied the intestinal flora of laboratory mice to detect, if possible, variation of flora associated with different degrees of infectivity, as well as any alteration in the normal flora following ingestion of the pathogenic bacilli.

Technique.

The mice employed were obtained from the stock breeding room. The regular diet of bread and milk was continued. Various quantitative methods for determining the intestinal flora led to the following technique.

Stool Cultures.—If the mouse, held in the usual way, 2 or 3 hours after feeding, was rubbed gently on the abdomen, the feces were voided. It was then possible to transfer a specimen to a sterile mortar with a platinum loop without fear of contamination. An emulsion was made with 5 cc. of saline and from this, subsequent higher dilutions were prepared. It was found impracticable to weigh the feces, so all counts are rough approximations of the total number of bacteria actually present. They do show accurately, however, the numerical ratio between various species of bacteria present in any given stool.

¹ Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.

² Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

Intestinal Cultures.—The mouse was asphyxiated with chloroform, opened, and the intestines removed to a sterile Petri dish. Approximately 1 cm. each of duodenum, ileum, cecum, colon, and sigmoid was then sectioned, slit longitudinally, and placed with sterile instruments in a test-tube containing 5 cc. of saline and a few glass beads. After thorough mixing, subsequent higher dilutions were prepared from these emulsions. An attempt was made to choose sections of intestine containing similar amounts of fecal material, but this variable and the uncertainty of the standard section of 1 cm. make the rough total estimates only of value in showing the numerical ratio between the various species of bacteria present in small and large intestine.

Determination of the Normal Flora.

For a preliminary survey of the flora, stool cultures from 50 normal mice were examined, as well as intestinal autopsy cultures from eight normal mice. Optimum media for general use were determined and there was gained a general insight into the species of bacteria associated with the intestinal tract.

Primary inoculation of fecal and intestinal suspensions into plain broth, dextrose broth, dextrose broth fermentation tubes, and milk, at hydrogen ion concentrations from pH 3 to 9, was tried with subcultures at varying intervals of time, to plates of plain agar, dextrose and lactose agar, agar at pH 3 to 9, with and without brilliant green or gentian violet. No specificity or inhibition phenomena proved sharp enough for practical use in this work. Therefore, subsequently, the suspensions, at various dilutions, were streaked directly to agar plates, pH 7.4, containing 0.5 per cent dextrose, and incubated 24 hours. 18 hour broth cultures picked from these plates were then examined microscopically and subcultured into semisolid carbohydrate media, lead acetate broth, litmus milk, gelatin, and media for the indole and Vosges-Proskauer reactions. Cultures falling into the *Salmonella* group were titrated against a serum prepared from the stock mouse strain of *Bacillus pestis caviae*. The final identity of each colony, therefore, was based upon the above technique. While it is realized that, for a most exact identification, this procedure should

be repeated at least twice for each strain, the frequent recurrence of species and the large amount of material examined made any such refinement of method impracticable and unnecessary.

For the cultivation of anaerobes, kidney ascitic fluid media,³ potato media,⁴ cooked meat media,⁵ and glucose blood agar plates were used. Non-spore-bearing anaerobes were not isolated. Since direct plating did not yield as good results as primary incubation, each suspension was inoculated into meat medium, incubated 3 days, heated 20 minutes at 80°C., inoculated into potato medium, incubated 2 days, and then plated. Numerical estimation was not possible by this method. The plates were incubated 4 days in Brown anaerobe jars,⁶ colonies were seeded to potato media, and after 48 hours, the pure cultures were examined microscopically and transferred to inspissated serum, litmus milk, and peptone carbohydrate media for identification.

From the above 58 mice, the following microorganisms were recognized. Spirochetal forms and vibrios were abundant in direct smears from cecum and colon; *Trichomonas muris*⁷ was occasionally seen in direct smears from the cecum; yeasts, variable in number, were usually present. Cocci, white and yellow, some of which liquefied gelatin, were present in small numbers, usually in the upper part of the intestinal tract. The following Gram-positive bacilli were isolated. *Bacillus acidophilus* and aerobic colonies of *Bacillus bifidus* were invariably found; *Bacillus lactis citreum*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus siccus* were occasionally present. Gram-negative bacilli were identified as follows: *Bacillus coli communis*, *Bacillus coli communior*, and *Bacillus acidii lactici* were frequently found; *Bacillus alkaligenes*, *Bacillus lactis aerogenes*, and *Bacillus neapolitanus* were occasionally present. One colony belonging to the Salmonella group was seen. Spore-bearing anaerobes were rarely found in normal stools. *Bacillus bifidus* was invariably present,

³ Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

⁴ Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1921-22, xix, 113.

⁵ Robertson, M., *J. Path. and Bact.*, 1915-16, xx, 327.

⁶ Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

⁷ Wenrich, D. H., *J. Morphol.*, 1921, xxxvi, 119.

TABLE I.

Mouse No.	Region.	Smear.			Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> .*	Colon† group.	Anaerobes.
		Spirochetes.	Vibrios.	Yeasts.				
9	Duodenum.	—	—	—	550§	90,000§	500§	—
	1:5; 1:50†	—	—	—	125	720,000	1,500	—
	Ileum.	—	—	—	125	720,000	1,500	—
	1:5; 1:50†	—	—	—	125	720,000	1,500	—
	Cecum.	++	++	+	5,000	18,000,000	300,000	—
	1:500; 1:5,000†	++	++	+	5,000	18,000,000	300,000	—
	Colon.	+	+	+	500	390,000	7,500	—
10	1:50; 1:500†	+	+	+	500	390,000	7,500	—
	Sigmoid.	+	+	+	500	400,000	7,500	—
	1:50; 1:500†	+	+	+	500	400,000	7,500	—
	Duodenum.	—	—	—	500	4,000	—	—
	Ileum.	—	—	—	500	60,000	5,000	—
	Cecum.	++	++	+	500	1,000,000	1,500,000	—
	Colon.	+	+	+	200	150,000	500,000	—
11	Sigmoid.	+	+	+	—	10,000	1,000,000	—
	Duodenum.	—	—	—	50	1,600	—	—
	Ileum.	—	—	—	100	25,000	—	—
	Cecum.	+	+	?	5,000	2,500,000	—	—
	Colon.	+	+	?	—	250,000	—	—
	Sigmoid.	—	—	—	10,000	100,000	—	—
	Duodenum.	—	—	—	1,500	2,500	100	—
12	Ileum.	—	—	—	—	120,000	150	—
	Cecum.	+	+	?	150,000	2,000,000	—	—
	Colon.	+	+	?	5,000	250,000	—	—
	Sigmoid.	+	+	?	2,000	3,000	—	—
	Duodenum.	—	—	—	500	1,000	—	—
	Ileum.	—	—	—	1,000	104,000	4,000	—
	Cecum.	++	++	+	12,500	75,000	200,000	—
13	Colon.	+	+	+	—	15,000	50,000	—
	Sigmoid.	—	—	—	500	100,000	150,000	—

* Aerobic colonies only.

† *B. coli communis*, *B. coli communior*, and *B. acidi lactici*.

‡ These same dilutions were employed for each animal.

§ The figures indicate the number of colonies per 5 cc. of original emulsion.

|| The dashes indicate less than 50 or 500 colonies per 5 cc. of original emulsion according to dilution.

TABLE I—*Concluded.*

Mouse No.	Region.	Smear.			Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> .*	Colon† group.	Anaerobes.
		Spirochetes.	Vibrios.	Yeasts.				
14	Duodenum.	—	—	—	100	1,500	—	—
	Ileum.	—	—	—	1,500	10,000	250	—
	Cecum.	—	—	—	5,000	1,000,000	—	—
	Colon.	—	—	—	5,000	375,000	—	—
	Sigmoid.	—	—	—	20,000	100,000	—	—
15	Duodenum.	—	—	—	5,000	5,000	—	—
	Ileum.	—	—	—	2,000	12,500	350	—
	Cecum.	++	++	+	5,000	500,000	500	—
	Colon.	+	+	+	2,500	200,000	10,000	—
	Sigmoid.	—	—	—	—	—	—	—
16	Duodenum.	—	—	—	2,000	30,000	—	—
	Ileum.	—	—	+	2,500	50,000	—	—
	Cecum.	+	+	+	5,000	2,700,000	—	—
	Colon.	+	+	+	2,500	240,000	—	—
	Sigmoid.	—	—	—	1,000	700,000	—	—

Bacillus welchii was occasionally seen, and rarely a few unidentified Gram-negative forms grew out on the anaerobic plates.

This list of protozoa and bacteria is misleading unless the various species are related by numerical data. The following quantitative method brought out sharply the predominating types and gave a more simple indication of the flora.

Eight mice, 4 months old, fed on a bread and milk diet, were autopsied by the technique described above. Cultures were taken from duodenum, ileum, cecum, colon, and sigmoid. Suspensions were then diluted sufficiently to give discrete colonies on the various plates: duodenal and ileal contents 1:5 and 1:50, cecal contents 1:500 and 1:5,000, and colon and sigmoid contents 1:50 and 1:500. This method, while eliminating entirely organisms present in very small numbers, gave striking and characteristic plates.

From Table I it may be seen that the predominating organisms in the intestinal tract of white mice fed on a bread and milk diet are (1) spirochetes, vibrios, and yeasts, most plentiful in the cecum;

(2) micrococci, usually white, liquefying and non-liquefying, distributed in small numbers throughout the intestine; (3) the colon group, consisting of *Bacillus coli communis*, *Bacillus coli communior*, and *Bacillus acidilactici*, more frequent than the cocci, most plentiful in the lower bowel; and (4) *Bacillus acidophilus* and *Bacillus bifidus*, invariably present, considerably outnumbering all other species, throughout the intestinal tract.

Intestinal Flora and Infectivity.

Having gained this general knowledge of the normal flora, we were then able to study its rôle in mouse typhoid infection.

Experiment.—Thirty-three mice, weighing approximately 18 gm. each, were divided into Series A and B, and placed in two separate cages. Series A (sixteen mice) was continued on the usual bread and milk diet for 5 days; Series B (seventeen mice) was fed, for 5 days, on raw beef muscle and water. On the 6th day each of the thirty-three mice was placed in a separate jar and stool cultures were taken (Table II). On the 7th day the animals of Series A were given by stomach tube 0.5 cc. of an 18 hour broth culture of *B. pestis caviae* diluted 1:100; sixteen of Series B received the same treatment; No. 17 of Series B was reserved as a control. The bread and milk diet was continued and administered daily to Series A throughout the duration of the experiment (60 days) while eight of Series B were changed from the meat diet to bread and milk 3 days after inoculation and the remaining eight of Series B were changed to bread and milk diet 7 days after inoculation. Stool cultures were taken on Series A at short intervals during the 60 days of observation. Table II compares the floræ of Series A and B fed on bread and milk and meat diets respectively. Table III gives the protocols of Series A, and Text-fig. 1 compares the duration of life of Series A and B.

From Table II it is apparent that the mice fed on bread and milk showed floræ in which *Bacillus acidophilus* was the predominating organism, far outnumbering the colon group, and that when the diet was changed to meat and water, the floræ changed character almost immediately. The Gram-negative bacilli increased rapidly and became the predominating group, with numerous putrefactive, protein-splitting, and liquefying organisms evident. *Bacillus welchii* was usually present. *Bacillus acidophilus* was greatly reduced in number and was frequently absent. Table II does not show this contrast in such an extreme form as two duplicate series which were fed raw meat for a longer period of time.

Text-fig. 1 shows no striking difference in the course of disease or duration of life in the two series (A and B). Animals with the

TABLE II.
Effect of Diet on Intestinal Flora.

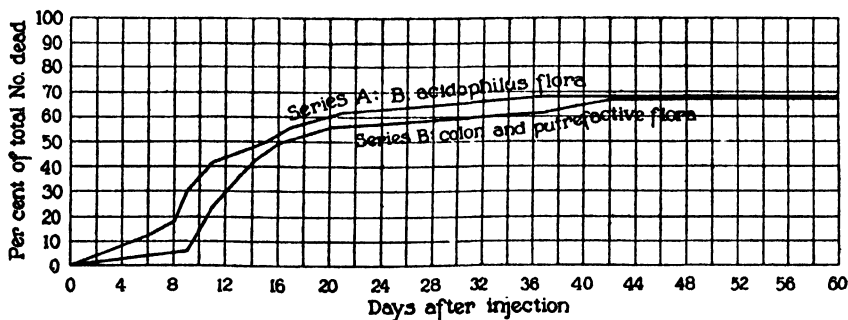
Series A.					Series B.				
Mouse No.	Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> (aerobic).	Colon group.*	<i>B. welchii</i> .	Mouse No.	Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> (aerobic).	Colon group.†	<i>B. welchii</i> .
1	—‡	2.5§	0.25	—	1	—	—	0.12	+
2	5.0	3.0	—	—	2	—	—	25.0	+
3	0.05	—	—	—	3	1.0	—	—	+
4	—	6.0	—	—	4	—	—	7.5	+
5	—	2.5	—	—	5	—	0.12	0.25	+
6	—	2.0	—	—	6	1.0	—	0.05	+
7	0.5	1.0	—	—	7	1.0	2.0	4.5	+
8	—	6.0	—	—	8	1.0	1.5	36.0	+
9	—	5.0	0.25	—	9	—	—	—	+
10	0.5	5.0	—	—	10	—	0.05	6.0	+
11	3.0	2.5	—	—	11	—	1.5	15.0	+
12	—	0.33	—	—	12	0.25	—	1.0	+
13	0.25	10.0	—	—	13	—	0.5	0.2	+
14	0.2	0.5	—	—	14	0.5	5.0	25.0	+
15	—	20.0	0.05	—	15	0.1	—	—	+
16	0.5	1.5	—	—	16	0.25	—	25.0	+
					17	0.02	—	3.0	+

* *B. coli*, *B. coli communis*, *B. coli communior*, and *B. acidi lactici*.

† *B. coli communis*, *B. coli communior*, *B. neapolitanus*, and *B. diffluens*.

‡ The dashes indicate less than 500 colonies per 5 cc. of original emulsion.

§ The figures indicate the number of colonies expressed in millions per 5 cc. of original emulsion.



TEXT-FIG. 1. Comparison of the duration of life of the mice of Series A and B.

TABLE III.
Protocols of Series A.

Mouse No.	Length of time after inoculation.	Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> (aerobic).	Colon group (<i>B. coli</i> and <i>B. acidilactici</i>).	Anaerobes.	<i>B. pestis</i> caviae.
	<i>days</i>					
1	1	—*	4.0†	—	—	0.05
	2	—	1.0	—	—	—
	3	—	2.0	—	—	—
	6	—	∞	—	—	0.0001
	8	—	0.0004	—	—	0.002
	14	—	0.2	0.05	—	0.04
	20	—	+ (?)	+ (?)	—	+ (?)
D. ‡§	22					
2	1	2.0	10.0	—	—	—
	2	—	9.0	—	—	—
	3	0.10	50.0	—	—	—
	8	—	∞	∞	—	—
D. §	11					
3	1	0.05	6.5	—	—	—
	2	—	6.0	—	—	—
	3	—	0.5	—	—	—
	6	3.0	12.5	—	—	—
	8	0.2	12.5	—	—	—
	14	—	—	—	—	∞
D. §	15					
4	1	—	6.0	—	—	0.05
	2	—	12.0	0.5	—	—
	3	—	20.0	0.1	—	—
	6	—	2.0	0.1	—	0.5
	8	—	—	—	—	—
D. §						
5	1	—	10.0	—	—	—
	2	—	10.0	—	—	—
	3	1.0	20.0	—	—	—
	6	—	10.0	—	—	—
	8	—	1.0	—	—	—
	14	—	50.0	5.0	—	—
	20	—	—	+ (?)	—	—
	35	—	—	—	—	—
D. §						

* The dashes indicate less than 500 colonies per 5 cc. of original emulsion.

† The figures indicate the number of colonies expressed in millions per 5 cc. of original emulsion.

‡ D. indicates dead, S. survived.

§ Autopsy lesions typical of mouse typhoid.

|| Heart's blood culture positive.

TABLE III—Continued.

Mouse No.	Length of time after inoculation.	Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> (aerobic).	Colon group (<i>B. coli</i> and <i>B. acidilactici</i>).	Anaerobes.	<i>B. pestis</i> <i>cavia</i> .
	<i>days</i>					
6	1	—	5.0	—	—	—
	2	—	12.5	—	—	—
	3	—	20.0	—	—	—
D.§	9	—				
7	1	—	1.2	—	—	—
	2	—	10.0	—	—	—
	3	—	25.0	—	—	—
	8	—	0.1	0.0001	—	0.001
	14	—	∞	∞	—	∞
D.§	17					
8	1	0.5	25.0	—	—	—
	2	—	35.0	—	—	—
	3	—	40.0	0.1	—	—
	6	0.5	2.0	0.005	—	—
	8	—	—	0.01	—	—
D.§	9					
9	1	—	50.0	7.5	—	—
	2	—	25.0	30.0	—	—
	3	—	15.0	7.5	—	—
	6	—	2.0	0.02	—	—
	8	—	—	0.0002	—	0.00002
	14	—	0.2	0.0005	—	0.0005
	20	+(?)	+(?)	+(?)	—	—
S.	60					
10	1	—	10.0	—	—	—
	2	—	5.0	—	—	—
	3	—	2.0	—	—	—
D.§	6					
11	1	—	—	—	—	—
	2	3.0	—	—	—	—
	3	—	—	—	—	1.0
	6	0.1	—	—	—	1.0
	14	—	—	—	—	—
	20	+(?)	—	+(?)	—	—
S.	60					

TABLE III—*Concluded.*

Mouse No.	Length of time after inoculation.	Cocci.	<i>B. acidophilus</i> and <i>B. bifidus</i> (aerobic).	Colon group (<i>B. coli</i> and <i>B. acidilactici</i>).	Anaero.	<i>B. pasteurianus</i> .
	<i>days</i>					
12	1	—	20.0	—	—	—
	2	—	2.0	—	—	—
	3	—	1.0	—	—	—
	6	—	1.0	—	—	—
	8	—	0.2	—	—	.25
	14	—	10.0	—	—	0
	20	+(?)	+(?)	+(?)	—	+(?)
S.	60					
13	1	—	25.0	—	—	—
	2	0.5	12.5	—	—	—
	3	0.05	10.0	0.04	—	—
	6	—	4.0	—	—	—
	8	—	0.2	—	—	—
D. §	11					
14	1	—	1.5	—	—	—
	2	—	20.0	0.15	—	—
	3	—	10.0	—	—	—
	14	—	10.0	—	—	—
	20	+(?)	+(?)	—	—	—
S.	60					
15	1	—	20.0	0.01	—	—
	2	—	4.0	—	—	—
	3	—	20.0	—	—	0.5
D. §	6					
16	1	1.5	16.0	—	—	—
	2	10.0	6.0	—	—	—
	3	5.0	10.0	0.02	—	—
	6	0.001	0.1	0.1	—	0.001
	8	0.005	0.2	—	—	0.005
	20	+(?)	+(?)	+(?)	—	—
S.	60					

acidophilus flora were as susceptible to the dose of mouse typhoid bacilli as were the animals with a colon and putrefactive flora. Two repetitions of this experiment gave similar results.

Finally, in the protocols of Series A (Table III) it may be seen that the surviving mice showed the same floræ as those of the susceptible mice which succumbed to the infection after varying intervals of time. In additional series of control mice, numbering about 75 in all, this same fact was constantly observed.

DISCUSSION.

Metchnikoff and his collaborators, studying the rôle of the intestinal flora, were impressed by the small numbers and few species of microbes inhabiting the gastrointestinal tract of mice and rats.^{8,9} Change of flora following change of diet was also emphasized by them,^{10, 11, 12} as well as by the more recent work of Torrey¹³ and Rettger and Cheplin¹⁴ who showed that in rats, dogs, and man, the intestinal flora bears a definite relation to the diet of the host, that lactose and dextrin have the power of stimulating growth of the aciduric bacteria, and that *Bacillus acidophilus*, administered *per os*, is capable of replacing the putrefactive and colon group.

While these facts have been very generally recognized, little credence has attended Metchnikoff's doctrine of orthobiosis, in which he stresses the importance of a putrefactive intestinal flora as a predisposing factor to gastrointestinal infection, as well as to many diseases of old age. Direct experimental evidence for this hypothesis has been meager;¹¹ nevertheless, Rettger, by attempting to alter the flora in this class of diseases, and as a prophylactic measure, seems to concede its validity.¹⁵

Our experiments show that in mice the intestinal flora plays no part in host susceptibility to mouse typhoid infection and that it does not affect the outcome of this disease.

⁸ Metchnikoff, Weinberg, Pozerski, Distaso, and Berthelot, *Ann. Inst. Pasteur*, 1909, xxiii, 937.

⁹ Tsiklinsky, *Ann. Inst. Pasteur*, 1914, xxviii, 441.

¹⁰ De Gasperi, F., *Centr. Bakt., 1te Abt., Orig.*, 1911, lvii, 519.

¹¹ Belonovsky, J., *Ann. Inst. Pasteur*, 1907, xxi, 991.

¹² Distaso, A., and Schiller, J., *Compt. rend. Soc. biol.*, 1914, lxvi, pt. 1, 179.

¹³ Torrey, J. C., *J. Med. Research*, 1918-19, xxxix, 415.

¹⁴ Rettger, L. F., and Cheplin, H. A., A treatise on the transformation of the intestinal flora with special reference to the implantation of *Bacillus acidophilus*, New Haven and London, 1921.

¹⁵ Rettger, L. F., and Cheplin, H. A., *Arch. Int. Med.*, 1922, xxix, 357.

CONCLUSIONS.

The normal flora of laboratory mice at The Rockefeller Institute, fed on a bread and milk diet, was determined. *Bacillus acidophilus* and *Bacillus bifidus* outnumber the *Bacillus coli*, *Bacillus acidilactici*, and *Bacillus coli communior* group about twenty-five to one. White and yellow cocci which may or may not liquefy gelatin are occasionally noted; spirochetal and vibrio forms and yeasts are usually seen in stained preparations. This flora does not change when mice are artificially infected *per os* with a strain of mouse typhoid bacilli (*Bacillus pestis caviæ*) and is the same in the animals which resist the infection as in those which succumb.

Mice fed on a meat diet and showing a colon, *Bacillus diffluens*, and *Bacillus welchii* flora do not differ in susceptibility to mouse typhoid from the normal mice fed on bread and milk and showing the above *acidophilus* flora.

OX BILE SENSITIZATION IN MOUSE TYPHOID INFECTION.

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(Received for publication, July 21, 1922.)

Earlier papers have emphasized the fact that in any series of laboratory mice a certain number is immune to mouse typhoid.¹ An explanation for this phenomenon was sought in an analysis of the biological environment at the normal portal of entry of the mouse typhoid bacilli, but it was found that the intestinal flora played no part in susceptibility to infection.² Attention was then directed to the intestinal wall itself in an effort to alter its permeability. Besredka, unable to infect mice with typhoid and paratyphoid bacilli, given by way of the mouth, found that sterile ox bile, administered 24 hours prior to the culture of organisms, rendered the animals susceptible to disease.³ Acting on this information, we administered ox bile to mice before injecting them *per os* with a strain of mouse typhoid bacilli (*Bacillus pestis caviæ*) and endeavored to discover and analyze any differences in susceptibility between the animals so treated and the controls.

The following experiment was devised to ascertain the proper dose of ox bile, the time for its administration, and the optimum interval before injecting the mice *per os* with the living cultures.

Experiment 1.—42 mice, weighing from 16 to 18 gm. each, were treated as follows: 18 mice, called Series A, received *per os*, by stomach tube, 0.5 cc. of undiluted sterile ox bile; 18, called Series B, received 0.25 cc. of bile made up to 0.5 cc. volume in salt solution; 6 were used as controls. 9 of Series A and 9 of Series B received the bile on an empty stomach and were fed 6 hours later; the remaining 9 of Series A and Series B received the bile $\frac{1}{2}$ hour after feeding.

¹ Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71.

² Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 21.

³ Besredka, A., *Ann. Inst. Pasteur*, 1918, xxxii, 193; 1919, xxxiii, 557, 882.

TABLE I.
Protocols of Mice Used in Experiment 1.

Mouse No.	Bile dosage. cc.	Time.	Condition of animal 24 hrs. after bile ingestion.	Time of inoculation.	Duration of life after inoculation. days
1	0.5	Full stomach.	Dead.	—	—
2	0.5	"	"	—	—
3	0.5	"	Severe diarrhea.	24 hrs. after bile treatment.	16
4	0.5	"	"	24 " " "	Survived.*
5	0.5	"	"	48 " " "	1
6	0.5	"	"	48 " " "	15
7	0.5	"	"	48 " " "	Survived.
8	0.5	"	"	Control.	5
9	0.5	"	"	"	5
10	0.5	Empty	Dead.	—	—
11	0.5	"	"	—	—
12	0.5	"	"	—	—
13	0.5	"	Severe diarrhea.	24 hrs. after bile treatment.	Survived.
14	0.5	"	"	24 " " "	"
15	0.5	"	"	48 " " "	6
16	0.5	"	"	48 " " "	6
17	0.5	"	"	Control.	5
18	0.5	"	"	"	Survived.

19	0.25	Full stomach.	Slight diarrhea.	24 hrs. after bile treatment.	5
20	0.25	" "	" "	24 " " "	6
21	0.25	" "	" "	24 " " "	Survived.
22	0.25	" "	" "	48 " " "	"
23	0.25	" "	" "	48 " " "	"
24	0.25	" "	" "	48 " " "	"
25	0.25	" "	" "	Control.	"
26	0.25	" "	" "	"	"
27	0.25	" "	" "	"	"
28	0.25	Empty	" "	24 hrs. after bile treatment.	12
29	0.25	" "	" "	24 " " "	Survived.
30	0.25	" "	" "	24 " " "	"
31	0.25	" "	" "	48 " " "	"
32	0.25	" "	" "	48 " " "	"
33	0.25	" "	" "	48 " " "	"
34	0.25	" "	" "	Control.	"
35	0.25	" "	" "	"	"
36	0.25	" "	" "	"	"
37	—	—	—	24 hr. series.	"
38	—	—	—	24 " " "	"
39	—	—	—	24 " " "	"
40	—	—	—	48 " " "	10
41	—	—	—	48 " " "	11
42	—	—	—	48 " " "	19

* Survivors discarded after 30 days.

TABLE II.

Protocols of Mice Used in Experiment 2.

Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutins.	Condition of animal.	Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutins.	Condition of animal.
A-1	1922						A-6	1922					
	Mar. 27	A = C	0	0	0	Well.		Mar. 27	A = C	0	0	0	Well.
	" 28	Bile ingested.						" 28	Bile ingested.				
	" 29	Bacilli ingested.						" 29	Bacilli ingested.				
	" 30	C	?	0	0	Well.		" 30	C, few A	++	++	0	Well.
	Apr. 1	" few A	+	+	—	"		Apr. 1	"	—	+	0	Diarr.
	" 3	" = A	+	+	—	"		" 3	C	0	++	0	Sick.
	" 6	" few A	+	++	—	Fair.		" 6	"	—	++	—	Sick.
A-2	" 8					D.*†	A-7	" 10					D.*†
	Mar. 27	A = C	0	0	0	Well.		Mar. 27	A, few c	0	0	0	Well.
	" 28	Bile ingested.						" 28	Bile ingested.				
	" 29	Bacilli ingested.						" 29	Bacilli ingested.				
	" 30	C	0	+	0	Diarr.		" 30	C, few A	++	0	0	Well.
	Apr. 1	" few A	0	0	—	"		Apr. 1	" = A	+	0	0	"
	" 3	" "	0	0	—	"		" 3	"	—	+	—	"
	" 6	" "	+	++	—	Fair.		" 6	"	—	+	—	Fair.
A-3	" 10					D.*†	A-8	" 10					D.*†
	Mar. 27	A few C	0	0	0	Well.		Mar. 27	A, few C	0	0	0	Well.
	" 28	Bile ingested.						" 28	Bile ingested.				
	" 29	Bacilli ingested.						" 29	Bacilli ingested.				
	" 30	C	0	0	0	Well.		" 30	C	0	0	0	Well.
	Apr. 1	" = A	0	0	—	"		Apr. 1	"	—	+	+	Well.
	" 3	" "	0	0	—	"		" 3	A = C	0	+	0	Sick.
	" 6	" "	0	0	—	"		" 5		0	++	—	Sick.

A-4	Apr. 12	A, few C	0	0	Well.	A-9	Mar. 27	A, few C	0	0	Well.
	" 18	"	0	0	"		" 28	Bile ingested.	0	0	Sl. diarr.
	" 25	" few c	0	0	"		" 29	Control.	0	0	Well.
	May 9	" C	0	0	"		" 30	C	0	0	"
	"	and c	0	0	"		Apr. 1	"	0	0	"
	" 29	A, few C	0	0	"		" 3	"	0	0	"
		and c	0	0	"		" 6	C, few A	0	0	"
	Mar. 27	A, few c	0	0	"		" 12	" = A	0	0	"
	" 28	Bile ingested.	0	0	"		" 18	A, few C	0	0	"
	" 29	Bacilli ingested.	0	0	"		" 25	" "	0	0	"
A-5	" 30	C	+	0	Well.		May 9	" "	0	0	"
	Apr. 1	A = C	0	0	"		" 29	" "	0	0	"
	" 3	" = "	0	0	"	A-10	Mar. 27	" c	0	0	"
	" 6	" = "	0	+	"		" 28	Bile ingested.	0	0	"
	" 12	" few C	0	0	"		" 29	Bacilli ingested.	0	+	Well.
	" 18	C, " A	0	0	"		" 30	C	0	0	"
	" 25	" "	0	0	"		Apr. 1	"	0	0	"
	May 4	" "	0	0	D.*†		" 3	"	0	+	"
			0	0	Well.		" 6	A, few c	0	+	Fair.
	Mar. 27	A = C	0	0	"	A-11	" 9		0	++	D.*†
A-5	" 28	Bile ingested.	0	0	Well.		Mar. 27	A, few C	0	0	Well.
	" 29	Bacilli ingested.	++	0	Well.		" 28	and c	0	0	
	" 30	C	++	0	Sick.		" 29	Bile ingested.	0	0	
	Apr. 1	"	+	0	D.†			Bacilli ingested.	0	0	
	" 2		+	0					0	0	

C indicates colon colonies; c, coccus colonies; A, *acidophilus* colonies; —, test not performed; 0, test negative; +, 1 to 10 colonies; ++, 10 to 50 colonies; +++ more than 50 colonies. D. indicates dead; Diarr., diarrhea; Sl. diarr., slight diarrhea.

* Autopsy lesions typical of mouse typhoid.

† Heart's blood culture positive.

TABLE II—Continued.

Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutinins.	Condition of animal.	Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutinins.	Condition of animal.
A-11	1922						B-4	1922					
	Mar. 30	C, few A	+	0	0	Well.		Mar. 27	A, few C	0	0	0	Well.
	Apr. 1	A	0	0	0	"		" 29	Bacilli ingested.				
	" 3	C = A	0	0	—	"		" 30	C, few A	0	0	0	Well.
	" 6	A, few c	0	0	—	"		Apr. 1	A, " C	0	0	0	"
	" 12	" "	0	0	0	"		" 3	" "	0	0	—	"
	" 18	" "	0	0	—	"		" 6	—	—	+	—	"
	" 25	" "	0	0	0	"		" 12	C, few A	0	0	0	Fair.
	May 9	" few c	0	0	0	"		" 18	" "	0	0	0	"
	" 15					D.*†		" 25	—	—	++	0	Sick.
A-12	Mar. 27	A = C	0	0	0	Well.	B-5	May 9	—	—	++	0	D.*†
	" 28	Bile ingested.						" 15	—	—			
	" 29	Control.				St. diarr.		Mar. 27	A = c	0	0	0	Well.
	" 30	C = A	0	0	0	Well.		" 29	Bacilli ingested.	++	0	0	Well.
	Apr. 1	" = "	0	0	—	"		" 30	A, few C and c	++	0	0	Well.
	" 3	A, few C	0	0	—	"	B-6	Apr. 1	A = c	0	0	0	"
	" 6	" = c	0	0	—	"		" 3	" few C	+	++	—	"
	" 12	Few C	0	0	0	"		" 6	—	—			D.*†
	" 18	A = c	0	0	0	"		Mar. 27	c	0	0	0	Well.
	" 25	Few C	0	0	—	"		" 29	Bacilli ingested.				
	" 30	A = c	0	0	—	"		" 30	A = c	0	0	0	Well.
	" 25	A = c	0	0	0	"		Apr. 1	" = "	0	0	0	"
	May 9	A = c	0	0	0	"		" 3	" "	0	0	—	"
	" 29	Few C	0	0	0	"		" 6	" few C	0	+	—	"
								" 12	" = C	+	++	0	Sick.
								" 13					D.*†

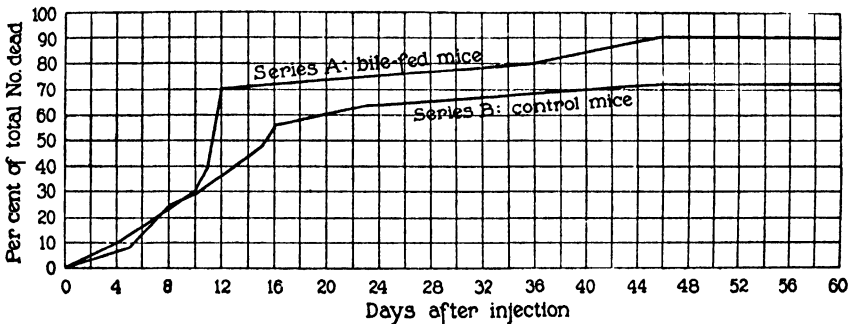
B-1	Mar. 27	A = C	0	0	Well.	B-7	Mar. 27	A = C	0	0	Well.
	" 29	Bacilli ingested.	++	0	Well.	B-8	" 29	Bacilli ingested.	0	0	Well.
	" 30	A = C	0	0	"		" 30	A, few C	+	0	"
	Apr. 1	" = C	0	0	"		Apr. 1	"	0	0	"
	" 3	" = C	0	0	"		" 3	"	0	0	"
	" 6	" few C	+	0	"		" 6	" few c	0	0	"
	" 12	—	—	0	Fair.		" 12	" = C	0	0	"
B-2	" 14	—	—	0	D.*†		" 18	" = "	+	0	Fair.
	Mar. 27	A, few C	0	0	Well.	B-9	" 21	" = "	+	0	D.*†
	" 29	Bacilli ingested.	0	0	Well.		Mar. 27	A = C	0	0	Well.
	" 30	A = C	0	0	"		" 29	Bacilli ingested.	0	0	Well.
	Apr. 1	—	—	0	"		" 30	A, few c	+	0	Well.
	" 3	—	—	0	"		Apr. 1	" C	0	0	"
	" 6	C	0	0	"		" 3	" "	0	0	"
	" 12	"	+	0	Sick.	B-10	" 6	"	0	0	D.*†
B-3	" 13	"	++	0	D.*†		Mar. 27	A, few C	0	0	Well.
	Mar. 27	A, few C	0	0	Well.		" 29	Bacilli ingested.	0	0	Well.
	" 29	Bacilli ingested.	0	0	Well.		" 30	A	0	0	Well.
	" 30	A, few C	0	0	"		Apr. 1	—	—	0	Sick.
	Apr. 1	"	0	0	"		" 3	—	—	—	"
	" 3	"	0	0	"		" 6	—	—	—	D.†
	" 9	"	+	0	"		" "	—	—	—	

TABLE II—*Concluded.*

Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutins.	Condition of animal.	Mouse No.	Date.	Type of flora.	Stool culture.	Blood culture.	Agglutins.	Condition of animal.
	1922							1922					
B-11	Mar. 27	C	0	0	0	Well.	B-12	Mar. 27	C	0	0	0	Well.
	" 29	Bacilli ingested.	0	0	—	Well.		" 29	Bacilli ingested.	+	0	0	Well.
	" 30	A = C	0	0	—	"		" 30	A = C	0	0	—	"
	Apr. 1	"	0	0	—	"		Apr. 1	"	0	0	—	"
	" 3	"	+	0	—	"		" 3	"	0	0	—	"
	" 6	" few C	0	0	—	"		" 6	" = C	0	0	—	"
	" 12	" "	0	0	0	"		" 12	" = "	0	0	0	"
	" 18	C	+	0	—	"		" 18	C	0	0	0	"
	" 25	A, few C	0	0	0	"		" 25	A, few C	0	0	0	"
	May 9	C	0	0	0	"		May 9	C	0	0	0	"
	" 29	" few A	0	0	0	"		" 29	" = A	0	0	0	"

24 hours later, 2 mice from Series A, which had ingested the bile on a full stomach, were dead; 3 mice from Series A, which had received the bile on an empty stomach, had succumbed; the survivors of Series A were extremely sick with signs of severe diarrhea. From Series B none had succumbed but all showed evidence of slight diarrhea.

At this time, 2 mice of Series A, which received the bile on a full stomach, were inoculated *per os*, by stomach tube, with a 1:200 dilution of an 18 hour broth culture, taken from the stock agar slant; 3 were injected the next day, 48 hours after the ox bile treatment; 2 were used as controls. 2 mice of Series A, which had received the bile on an empty stomach, were given a similar dose of bacteria, 2 were injected the next day, and 2 were used as controls. In the same manner, 6 mice of Series B were injected 24 hours and 6, 48 hours after the bile treatment, while 6 were saved for controls. The original controls were similarly injected. Table I shows the division of mice and subsequent series of events.



TEXT-FIG. 1. Experiment 2. Comparison of the mortality curves of the mice of Series A and B.

From this preliminary experiment it was concluded that 0.25 cc. of bile per mouse was a maximum dose, that the bile might be administered on a full or empty stomach, and that the mouse might be injected 24 or 48 hours after the bile treatment, without grossly altering the course of disease.

Experiment 2.—24 mice, averaging 18 to 20 gm., were placed in separate jars and stool cultures were taken. The next day, 12 mice (Series A) received *per os* 0.25 cc. of sterile ox bile, made up to a volume of 0.5 cc. 24 hours later, 10 of these mice and the 12 remaining controls (Series B) received *per os* a 1:200 dilution of an 18 hour broth culture of *B. pestis caviæ* taken from the stock tube. 2 mice from Series A were saved as controls. The condition of the animals was noted. Stool cultures and blood cultures were taken and the blood was examined for agglutinins at short intervals. The technique for stool culturing has been de-

scribed.² Blood cultures were made by clipping the tail and expressing 3 drops to a 0.5 per cent dextrose agar plate. The technique used in agglutination tests has been described.⁴ Table II shows the protocols for each mouse and Text-fig. 1 shows the mortality curves of Series A and B.

This experiment shows that ox bile, administered in doses large enough to cause diarrhea, increases the susceptibility of mice to mouse typhoid infection by slightly raising the death rate and shortening somewhat the duration of life of the susceptible animals.

DISCUSSION.

The above observations are of value in interpreting the recent work of Besredka on intestinal immunity to experimental typhoid, paratyphoid, and dysentery infection. He found that ox bile, given *per os* to laboratory animals, not only rendered them susceptible to enteric infection by this route but increased the efficacy of gastrointestinal immunization against a subsequent injection of the homologous strain *per os* or intravenously. We found that ox bile increased the susceptibility of mice to mouse typhoid infection only when given in maximum doses. It then seemed to raise the death rate and shorten the duration of life of the susceptible animals.

According to Besredka, bile injures the intestinal epithelium, allowing the pathogenic bacilli to gain easy access to the blood stream. And judging from the evident discomfort and diarrhea following bile ingestion in our animals, it seems quite probable that his interpretation is correct. But while host susceptibility to typhoid infection may be altered by injury to the intestinal epithelium, experiments so far have failed to justify his claim that all immunity to typhoid, paratyphoid, and dysentery infection, natural or acquired, is localized in the intestinal tract. On the contrary, in individual susceptibility⁵ and in acquired immunity to mouse typhoid infection,¹ the intestinal wall is but one factor in a general mechanism.

CONCLUSION.

Sterile ox bile, administered *per os* in maximum doses to laboratory mice, tends to increase their susceptibility to mouse typhoid infection.

² Amoss, H. L., *J. Exp. Med.*, 1922, **xxxvi**, 45.

⁵ Webster, L. T., *J. Exp. Med.*, 1923, **xxxvii** (in press).

STROPHANTHIN.

I. STROPHANTHIDIN.

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(Received for publication, July 27, 1922.)

The classical studies of Fraser¹ and the confirmatory work of Feist² on the Kombé arrow-poison have shown it to contain a glucoside, strophanthin, which yields on hydrolysis a crystalline substance, strophanthidin, and reducing sugars. Although Fraser worked at a time when botanical classification was uncertain, it is now conceded that his material was in the main *Strophanthus kombé*. More recent comparative studies³ have demonstrated that the glucosides of *Strophanthus kombé* and *Strophanthus hispidus* are closely related since they yield the same strophanthidin on hydrolysis. More recently Windaus and Hermanns⁴ have found that the cymarin of *Apocynum cannabinum* is also a glucoside of strophanthidin (cymarigenin). Strophanthidin is thus seen to be a component of a number of glucosides which possess a characteristic action on the heart, and further study of its still obscure chemistry is of interest especially since there is suspicion of its close relationship to the still little understood members of the digitalis series.

Feist, who was the first to attempt a careful inquiry into the nature of the substance, deduced the formula $C_{27}H_{38}O_7 + 2\frac{1}{2} H_2O$. The difficulty which he experienced in preparing the anhydrous substance

¹ Fraser, T. R., *Strophanthus hispidus*; its natural history, chemistry, and pharmacology, Edinburgh, 1891. Fraser, T. R., and Dobbin, L., *Tr. Roy. Soc. Edinburgh*, 1891-93, xxxvii, 1.

² Feist, F., *Ber. chem. Ges.*, 1898, xxxi, 534; *Ber. chem. Ges.*, 1900, xxxiii, 2063, 2069, 2091.

³ Heffter, A., and Sachs, F., *Biochem. Z.*, 1912, xl, 83. Brauns, D. H., and Closson, O. E., *J. Am. Pharmaceut. Assn.*, 1913, ii, 489, 604, 715.

⁴ Windaus, A., and Hermanns, L., *Ber. chem. Ges.*, 1915, xlviii, 979, 991.

by ordinary means was overcome by the preparation of a form containing methyl alcohol of crystallization which was readily driven off. The lactone nature of the substance was determined by its behavior toward alkali. The resulting acid when liberated was not isolated as such but lost water with the formation of a new crystalline isomeric lactone which he called strophanthidinic acid lactone. The only other definite product studied by him was a crystalline strophanthic acid which resulted from the oxidation of strophanthidin with permanganate in alkaline solution and for which he adopted the incorrect formula $C_{27}H_{38}O_9$.

On the basis of analysis and titration of strophanthidin as well as data obtained from the glucoside, cymarín, itself, Windaus and Hermanns have more recently derived the formula $C_{23}H_{30}O_5 + 1\frac{1}{2} H_2O$ for the air-dry substance. They likewise experienced difficulty in obtaining the anhydrous substance but since long drying *in vacuo* at 110° caused a loss of water corresponding to $1\frac{1}{2}$ mols, they concluded that it has, when anhydrous, the composition, $C_{23}H_{30}O_5$. Contrary to these workers we have experienced little difficulty in drying the substance to constant weight *in vacuo* at 110° over phosphorus pentoxide and we have noted a loss in weight corresponding to only $\frac{1}{2} H_2O$. Similar observations have already been recorded by Brauns and Closson,³ who, however, accepted the older formula of Feist. Our analytical data force the conclusion that anhydrous strophanthidin possesses the formula $C_{23}H_{32}O_6$ and that the loss in weight corresponding to $1\frac{1}{2} H_2O$ noted by Windaus and Hermanns was in part the result of decomposition. This conclusion is supported by the analysis of strophanthidin benzoate which these workers reported and which we have confirmed, and also by the analytical data obtained with all the derivatives to be described below. By way of confirmation of the choice between the C_{23} formula and the original C_{27} formula of Feist we believed that the preparation and complete analysis of the *p*-bromobenzoate should prove quite conclusive. The result confirmed the formula $C_{23}H_{32}O_6$.

On reduction with palladium and hydrogen, strophanthidin yielded a dihydro compound, $C_{23}H_{34}O_6$, which was isolated as a mono- and a dihydrate. Although the reaction required an unusual time for completion and a relatively large amount of colloidal palladium, it

seems probable that the point of attack was a double bond. The reduction of a carbonyl group seems excluded since only a mono-benzoate was obtained by benzylation of the hydrogenated compound. Contrary to Feist and Fraser, Windaus and Hermanns state that strophanthidin reacts with ketone reagents, but they were unable to isolate definite products. We have on the contrary prepared the crystalline oxime and the phenyl and *p*-bromophenylhydrazones of this substance which definitely prove the presence of a carbonyl group. From all indications this is ketonic in character.

Finally, our analyses show that isostrophanthidin (isocymarigenin of Windaus and Hermanns or strophanthidinic acid lactone of Feist), like strophanthidin, possesses when anhydrous the formula $C_{23}H_{32}O_6$. In accord with this the formula of isostrophanthidin benzoate was found to be $C_{30}H_{38}O_7$.

At present it may be concluded that strophanthidin, although it may have at least one double bond, possesses mainly a saturated alicyclic skeleton. Of the six oxygen atoms, two are accounted for by the lactone group, one by a carbonyl group, and one by an alcoholic group.

EXPERIMENTAL.

Strophanthidin.—*Strophanthus kombé* seeds, purchased on the open market, were ground, defatted with gasoline and then exhaustively percolated with 70 per cent alcohol. The resulting extract was concentrated under diminished pressure to a thick syrup⁵ which was then worked up in portions of 500 gm. In each case this amount was dissolved in 1 liter of warm water. The solution was cooled and treated with basic lead acetate solution as long as a precipitate formed. At first crude strophanthin was precipitated from the filtrate and washings with an excess of solid ammonium sulfate according to Thoms,⁶ but this added purification was soon found to be unnecessary for the preparation of strophanthidin. Instead the above filtrate and washings were treated with sufficient 25 per cent sulfuric acid to precipitate the dissolved lead and then filtered. Concen-

⁵ The large scale operations were performed for us by Schieffelin and Co. of New York.

⁶ Thoms, H., *Ber. pharm. Ges.*, 1904, xiv, 104.

trated hydrochloric acid was added to the filtrate until the latter became just acid to Congo red, an excess of acid being carefully avoided. The mixture, the volume of which was about 1,500 cc., was then heated on the water bath for 3 to 4 hours at 70–80°C. The olive-colored solution slowly deposited the crude strophanthidin as lustrous plates which were somewhat tinged with green. The collected crystals were washed with water and air-dried. The yield of crude product was about 25 gm. from each kilo of seeds.

When recrystallized from 95 per cent alcohol the substance was readily purified and formed stout rhombic crystals which agreed essentially in all properties with those recorded in the literature. Its specific rotation was found to be

$$[\alpha]_D^{25} = + 43.1^\circ \text{ (} c = 2.796 \text{ in methyl alcohol.)}$$

As a rule it melted with effervescence at about 171–175° although the exact point depended upon the rate of heating. Occasionally we have obtained crystals which scarcely sintered at this point and melted at about 230°. In either case the air-dried material contained 0.5 mol of water of crystallization which it gradually lost when heated under 20 mm. pressure at 100–110° over phosphorus pentoxide, with accompanying slight discoloration. Contrary to Windaus and Hermanns⁴ no further loss of water was observed on continued heating even at a pressure below 1 mm. except in cases where sulfuric acid was used as the drying agent. Here extensive decomposition occurred, as shown by the formation of a dark brown resin which no longer had the physical properties of strophanthidin. Brauns and Closson³ likewise found no difficulty in removing the water of crystallization which they considered to be 1 molecule on the basis of Feist's² old formula, $C_{27}H_{38}O_7$. Our analytical data, as well as those of Brauns and Closson, obtained with anhydrous strophanthidin, agree just as well for the formula $C_{23}H_{32}O_6$ which we adopt.

Air-dry substance.

$C_{27}H_{38}O_7$.	$\frac{1}{2} H_2O$.	Calculated.	C 66.79, H 8.05.
		Found, (a).	" 66.65, " 7.91.
		" (b).	" 66.59, " 7.82.

Anhydrous substance.

$C_{23}H_{32}O_6$.	Calculated.	C 68.27, H 7.98.
	Found, (a).	" 68.24, " 8.12.
	" (b).	" 68.14, " 8.18.

Strophanthidin Benzoate.—This was prepared essentially according to the method used by Windaus and Hermanns. To the properties recorded by these workers we may add that the substance is easily soluble in chloroform and acetone and sparingly so in the cold in methyl and ethyl alcohols. In concentrated sulfuric acid it gives an orange-red color which changes to a brown-orange.

Our analysis of this substance dried *in vacuo* at 110° accords with the results of Windaus and Hermanns and gives the formula $C_{30}H_{36}O_7$. The air-dry substance, however, contained $1\frac{1}{2}H_2O$ and gave

$$[\alpha]_D^{20} = +47.8^\circ \quad (c = 1.067 \text{ in acetone.})$$

Air-dry substance.

$C_{30}H_{36}O_7 \cdot 1\frac{1}{2}H_2O$.	Calculated.	H_2O	5.05.
	Found.	"	4.68.

Anhydrous substance.

$C_{30}H_{36}O_7$.	Calculated.	C	70.83,	H	7.14.
	Found, (a).	"	70.70,	"	7.28.
		"	(b).	"	70.66, " 7.20.

Strophanthidin p-Bromobenzoate.—2 gm. of strophanthidin were dissolved in 30 cc. of pyridine and the chilled solution was treated with 2 gm. of *p*-bromobenzoyl chloride dissolved in ether. After 24 hours the ester was precipitated with water. After digesting the collected precipitate with dilute carbonate it was filtered off and washed with water. The careful addition of water to the solution in hot acetic acid caused the separation of a rapidly crystallizing gum. The air-dried substance is sparingly soluble and contains 1 molecule of water of crystallization which is readily removed *in vacuo* at 110°. The anhydrous substance melts at 222–224° with decomposition and gives

$$[\alpha]_D^{20} = +42.0^\circ \quad (c = 1.094 \text{ in acetone.})$$

The analysis of this substance makes possible a definite and final decision between the formula $C_{30}H_{36}O_7Br$ based on that for strophanthidin of $C_{23}H_{32}O_6$ and the one based on the formula of Feist; *i.e.*, $C_{34}H_{41}O_8Br$.

Air-dry substance.

$C_{30}H_{38}O_7Br \cdot H_2O$.	Calculated.	H_2O	2.98.
	Found.	"	3.53.

Anhydrous substance.

$C_{30}H_{38}O_7Br$.	Calculated.	C	61.31,	H	6.01,	Br	13.61.
$C_{34}H_{44}O_8Br$.	"	"	62.08,	"	6.29,	"	12.16.
	Found, (a).	"	61.07,	"	6.02,	"	13.57.
	" (b).	"	61.25,	"	6.04,	"	13.73.

Strophanthidin Oxime.—The oxime was readily obtained by boiling for several hours a solution of equivalent amounts of strophanthidin and hydroxylamine hydrochloride in alcohol containing sufficient sodium acetate. Recrystallized from alcohol it forms minute, colorless, glistening prisms which melt and slowly effervesce at 270–275°. It is easily soluble in pyridine and dissolves less readily in alcohol and acetic acid. In concentrated sulfuric acid the solution is at first yellow and changes through orange to a red shade.

$$[\alpha]_D^{25} = +71.3^\circ \quad (c = 1.009 \text{ in pyridine.})$$

$C_{22}H_{33}O_6N$.	Calculated.	C	65.83,	H	7.93.
	Found, (a).	"	65.95,	"	7.98.
	" (b).	"	66.12,	"	7.92.

Strophanthidin Phenylhydrazone.—A solution of 2 gm. of strophanthidin in 20 cc. of absolute alcohol was treated with 1 gm. of phenylhydrazine. On allowing the mixture to evaporate spontaneously at room temperature a crop of large stout prisms formed. Recrystallized from 95 per cent alcohol the hydrazone separates as colorless, glistening, well formed prisms which sinter slightly at about 175° and melt at 230–232° to a liquid filled with bubbles. It dissolves readily in chloroform, acetone, and alcohol, and but sparingly in ether or benzene. In concentrated sulfuric acid it gives a greenish yellow color which changes through brown to red and finally to purple.

$$[\alpha]_D^{25} = -5.0^\circ \quad (c = 1.000 \text{ in chloroform.})$$

Air-dry substance (dried at 100° *in vacuo* over H_2SO_4).

$C_{28}H_{38}O_6N_2 \cdot 2H_2O$.	Calculated.	H_2O	6.79.
	Found.	"	6.88.

Anhydrous substance.

$C_{28}H_{38}O_6N_2$.	Calculated.	C	70.42,	H	7.75,	N	5.67.
	Found, (a).	"	70.30,	"	7.60,	"	5.91.
	" (b).	"	70.34,	"	7.56,	"	6.01.

Strophanthidin p-Bromophenylhydrazone.—Equimolecular amounts of strophanthidin and *p*-bromophenylhydrazine were dissolved in a small volume of acetic acid. Shortly, on rubbing, crystallization started, the hydrazone forming glistening, colorless rhombs which retained solvent of crystallization. When recrystallized from methyl alcohol the acetic acid in the compound is replaced by the alcohol, yielding small pointed prisms which soften to a vitreous mass from 180–185° and are not completely molten until 200° is reached. The substance dissolves easily in alcohol and acetone, less readily in chloroform and ether, and very sparingly in benzene, forming a gum before dissolving in these solvents. In concentrated sulfuric acid it dissolves with a light brown color which changes first to red, then to a dirty purple, and finally becomes green.

$$[\alpha]_D^{25.5} = +105.5^\circ \text{ (} c = 1.004 \text{ in chloroform.)}$$

Air-dry substance (dried at 100° *in vacuo* over H₂SO₄).

$$\text{C}_{29}\text{H}_{37}\text{O}_6\text{N}_2\text{Br} \cdot 1\frac{1}{2}\text{CH}_3\text{OH. Calculated. CH}_3\text{OH 7.72.}$$

$$\text{Found. " 7.70.}$$

Anhydrous substance.

$$\text{C}_{29}\text{H}_{37}\text{O}_6\text{N}_2\text{Br. Calculated. C 60.71, H 6.51, N 4.89, Br 13.93.}$$

$$\text{Found, (a). " 60.78, " 6.42, " 5.18, " 13.73.}$$

$$\text{" (b). " 60.96, " 6.42.}$$

Dihydrostrophanthidin.—15 gm. of carefully purified strophanthidin were dissolved in 300 cc. of pure methyl alcohol, treated with a solution of 0.5 gm. of colloidal palladium in 50 per cent methyl alcohol, and shaken in a reduction apparatus in an atmosphere of hydrogen. Absorption of the gas, which occurred at the rate of 20 cc. for the 1st hour, rapidly decreased to a rate of 5 to 8 cc. per hour. After 3 days, an additional 0.5 gm. of colloidal palladium was added. This caused, however, only a temporary acceleration of the hydrogen absorption. After 2 weeks this had practically ceased, a total of 945 cc. having been absorbed, an amount which is not far removed from 1 mol, especially where leakage over so long a period was unavoidable. The solution was acidified with a few drops of acetic acid and the coagulated palladium was filtered off. After boiling off the alcohol from the filtrate a mixture of oil and water remained which was boiled

with 1 liter of water and a little bone-black. The filtered solution formed an emulsion which slowly crystallized when allowed to cool and be rubbed. After collecting, washing with water, and drying, the yield was 12 gm. Recrystallized from a small volume of 50 per cent alcohol, the substance separates as a dihydrate in the form of glistening rhombic prisms. It melts and effervesces at 100–103°C, then resolidifies and again melts with effervescence at 145–147° or merely sinters at this point and then melts at about 190–195°, depending upon the rate of heating. It dissolves readily in alcohol, acetone, and acetic acid, not so easily in chloroform, hot water, and hot benzene, and but sparingly in ether. In methyl alcohol

$$[\alpha]_D^{25} = + 34.85^\circ \quad (c = 1.004).$$

In concentrated sulfuric acid dihydrostrophanthidin gives a bright red-orange color of a much brighter, clearer, and permanent tint than that given by strophanthidin. The latter gives at first a dirty orange with a greenish tinge, later becoming a deep brown-red. Although it retains its water of crystallization even after long exposure to air, the substance loses this completely in a few hours at room temperature in a vacuum desiccator over sulfuric acid. The resulting product melts at 190–195° and is anhydrous since it loses but little more when heated *in vacuo* at 110° over phosphorus pentoxide.

Air-dry substance (dried at first at room temperature and finally at 100° *in vacuo* over H₂SO₄).

$$\text{C}_{23}\text{H}_{34}\text{O}_6 \cdot 2\text{H}_2\text{O}. \quad \text{Calculated.} \quad \text{H}_2\text{O} \ 8.14.$$

$$\text{Found.} \quad \quad \quad \text{"} \ 8.17.$$

Anhydrous substance.

$$\text{C}_{23}\text{H}_{34}\text{O}_6. \quad \text{Calculated.} \quad \text{C} \ 67.99, \text{H} \ 8.44$$

$$\text{Found.} \quad \quad \quad \text{"} \ 68.20, \text{"} \ 8.43.$$

A different hydrate was also obtained by maintaining an aqueous solution near the boiling point for some time with occasional rubbing. Characteristic rhombs were formed containing 1 molecule of water of crystallization. These were dissolved in a small volume of warm 50 per cent alcohol, treated with hot water to turbidity, and seeded. This form, when rapidly heated, melts and effervesces at 145–147°, and then resolidifies. If the rate of heating is too slow it merely

sinters at this point and melts at 190–195°. In solubilities it resembles the dihydrate with the exception of its lower solubility in hot water.

Air-dry substance (dried at 100° *in vacuo* over H_2SO_4).

$\text{C}_{23}\text{H}_{34}\text{O}_6 \cdot \text{H}_2\text{O}$. Calculated. H_2O 4.24.
Found. " 4.01.

Anhydrous substance.

$\text{C}_{22}\text{H}_{32}\text{O}_6$. Calculated. C 67.99, H 8.44.
Found. " 68.02, " 8.37.

Dihydrostrophanthidin Benzoate.—This substance was prepared like the corresponding strophanthidin derivative. Recrystallized from 85 per cent alcohol it forms minute glistening prisms which melt with effervescence at 225–227° and are soluble in chloroform and acetic acid and less readily so in alcohol and benzene.

$\text{C}_{30}\text{H}_{38}\text{O}_7$. Calculated. C 70.55, H 7.50.
Found, (a). " 70.70, " 7.51.
" (b). " 70.73, " 7.29.

Iso-strophanthidin.—(Strophanthidinic acid lactone of Feist, isocymarigenin of Windaus and Hermanns.) This was prepared in about 50 per cent yield by a method which was essentially that used by Windaus and Hermanns for the preparation of isocymarigenin. The air-dry material contains water, which, contrary to these workers, is readily removed on drying the substance *in vacuo* at 100° over phosphorus pentoxide. The analytical data indicate that the air-dry material possesses the same formula as strophanthidin, $\text{C}_{23}\text{H}_{32}\text{O}_6 \cdot \frac{1}{2} \text{H}_2\text{O}$.

$[\alpha]_D^{25} = +36.2^\circ$ ($c = 0.705$ in methyl alcohol.)

Air-dry substance.

$\text{C}_{23}\text{H}_{32}\text{O}_6 \cdot \frac{1}{2} \text{H}_2\text{O}$. Calculated. H_2O 2.18.
Found. " 1.78.

Anhydrous substance.

$\text{C}_{22}\text{H}_{32}\text{O}_6$. Calculated. C 68.27, H 7.98.
Found, (a). " 68.49, " 8.04.
" (b). " 68.75, " 7.88.

Isostrophanthidin Benzoate.—Isostrophanthidin was benzoylated as in previous cases. When recrystallized from alcohol, in which it is very sparingly soluble, the benzoyl derivative forms rosettes of microscopic leaflets which melt with preliminary sintering at about 270° when rapidly heated. It is soluble in chloroform and acetone, less readily in acetic acid.

$$[\alpha]_D^{25} = +38.0^\circ \text{ (} c = 1.002 \text{ in chloroform.)}$$

$C_{38}H_{38}O_7$. Calculated. C 70.83, H 7.14.

Found. " 70.98, " 7.08.

ON OXIDATION OF TERTIARY HYDROCARBONS.

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(Received for publication, June 2, 1922.)

Hydrocarbons with a tertiary carbon atom have attracted little attention. Few have been prepared synthetically and of those occurring in nature only a few have been investigated from the viewpoint of their structures. The lack of interest in this group of substances may be due to the belief in their inertness. However, there is accumulating evidence to show that the lack of reactivity of the aliphatic hydrocarbons is not as great as accepted in the past, particularly in the case of the hydrocarbons with a tertiary carbon atom. The fact that 3-methyl-pentane is capable of decolorizing a solution of potassium permanganate has been known since 1901.¹ The products of oxidation of this substance were not analyzed.

A considerable number of naturally occurring substances, among which are the higher fatty acids, are derivatives of tertiary hydrocarbons. The knowledge of their structures depends on that of the structures of the hydrocarbons.

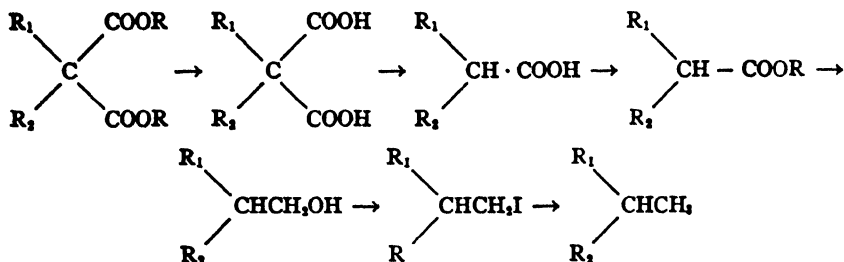
The present work was undertaken in 1918 in coöperation with L. H. Cretcher. The work was interrupted due to the conditions of war and at that time a short publication on the subject was published in this Journal.² Subsequently, the work was continued by the present writers.

Recently, the subject of oxidation of saturated hydrocarbons attracted considerable attention. However, on the special problem of oxidation of tertiary hydrocarbons no additional work has been reported.

¹ Zelinsky, N., and Zelikow, J., *Ber. chem. Ges.*, 1901, xxxiv, 2865.

² Levene, P. A., and Cretcher, L. H., Jr., *J. Biol. Chem.*, 1918, xxxiii, 505.

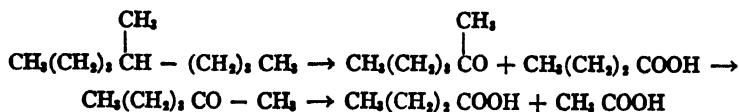
The present communication contains a report on the synthesis of five tertiary hydrocarbons. Their properties are tabulated below. The method of preparation was the same as employed in the previous work; namely, through the malonic ester synthesis by the following series of reactions:



This tedious method of preparation was adopted because paraffins are freed from impurities with great difficulty and therefore can be obtained in a sufficient degree of purity best by a set of reactions each simple in its nature and when each intermediary substance can be isolated and purified.

Turning to the oxidation by means of permanganate solution it was found that for 5-methyl-nonane the conditions employed by Levene and Cretcher were the most favorable. The higher tertiary hydrocarbons, however, were not oxidized under identical conditions. The failure may be due to the lowering in solubility of the hydrocarbons with increase of their molecular weights. The conditions for the oxidation of substances with higher molecular weights will have to be worked out.

Among the acids formed on oxidation of 5-methyl-nonane, two were identified; namely, acetic and butyric acids. The presence of some formic acid was detected. On the basis of this, the oxidation seems to progress in the main as follows:



General conclusions as to the progress of oxidation of tertiary hydrocarbons will be reached only after the observations have been extended to a greater number of these substances.

Hydrocarbons.

No.	Hydrocarbon.	Boiling point.		D_4^{20}	N_D^{20}	M_D^{20}	
		Temperature.	Pressure.			Calculated.	Found.
		°C.	mm.				
1	3-Methyl-heptane.	120-122	755	0.7069	1.3980	39.14	38.92
2	3-Methyl-nonane.	165.5-166.5	751	0.7354	1.4126	48.38	48.12
3	5-Methyl-nonane	164-166	755	0.7319	1.4116	48.38	48.24
4	5-Propyl-nonane.	204-205	763	0.7559	1.4228	57.62	57.25
5	5-Methyl-dodecane.	225.5-227	758	0.7576	1.4244	62.23	62.02

EXPERIMENTAL.

All melting and boiling point temperatures recorded in the following experiments were determined with standardized Anschütz thermometers.

Diethyl Ethyl-Malonnate.—Ethyl iodide was condensed with malonic ester in the presence of sodium ethylate as described by Conrad.³ The yield of substituted ester was 83 per cent of the theoretical. It boiled at 206–208°C.

Diethyl Butyl-Ethyl-Malonnate.—This ester was prepared by the condensation of butyl iodide and diethyl ethyl-malonnate. Sodium (12.2 gm.) was dissolved in absolute alcohol (150 cc.) and the diethyl ethyl-malonnate added. Butyl iodide (112 gm.) was then cautiously introduced and the solution boiled on the water bath over night. After the solution had become neutral, most of the alcohol was removed by distillation. The residue was taken up in ether, filtered to remove the sodium iodide, and distilled. The ester boiled at 128–129°C. at 7 mm. The yield was 62 per cent of the theoretical. Although prepared earlier by Raper⁴ the ester had not been characterized.

$$D_4^{20} = 0.9646$$

$$N_D^{20} = 1.4284$$

0.0990 gm. substance: 0.2312 gm. CO_2 and 0.0874 gm. H_2O .

$\text{C}_{13}\text{H}_{24}\text{O}_4$. Calculated. C 63.93, H 9.84, M_D^{20} 65.54.

Found. " 63.68, " 9.88, " 65.13.

³ Conrad, M., *Ann. Chem.*, 1880, cciv, 134.

⁴ Raper, H. S., *J. Chem. Soc.*, 1907, xci, 1837.

Butyl-Ethyl-Malonic Acid.—For the preparation of the acid, the above ester was not isolated but was saponified by heating the solution in which it was formed with a large excess of sodium hydroxide. After crystallization from high boiling gasoline, the acid melted at 115°C.

0.1020 gm. substance: 0.2168 gm. CO₂ and 0.0798 gm. H₂O.

C₉H₁₈O₄. Calculated. C 57.44, H 8.51.

Found. " 57.96, " 8.74.

2-Ethyl-Hexylic Acid.—Butyl-ethyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved and the residue distilled. It boiled at 228–229°C. at 755 mm. Raper⁴ found a boiling point of 225°C.

0.1020 gm. substance: 0.2500 gm. CO₂ and 0.1028 gm. H₂O.

C₈H₁₆O₂. Calculated. C 66.66, H 11.11.

Found. " 66.83, " 11.28.

Ethyl 2-Ethyl-Hexylate.—The acid was esterified in the usual manner with absolute alcohol and sulfuric acid. The yield was 91 per cent of the theoretical. The ester boiled at 189–191°C. at 766 mm.

$$D_4^{20} = 0.8628$$

$$N_D^{20} = 1.4128$$

0.1006 gm. substance: 0.2566 gm. CO₂ and 0.1040 gm. H₂O.

C₁₀H₂₀O₂. Calculated. C 69.76, H 11.62, M_D²⁰ 50.03.

Found. " 69.56, " 11.56, " 49.69.

2-Ethyl-Hexanol.—Ethyl 2-ethyl-hexylate was reduced by means of sodium and absolute alcohol. The method has been described by Levene and Cretcher.² The yield of alcohol was 78 per cent of the theoretical and in addition to this 10 per cent of the ester was recovered as 2-ethyl-hexylic acid. The alcohol boiled at 181–183°C. at 743 mm.

$$D_4^{20} = 0.8328$$

$$N_D^{20} = 1.4328$$

0.1016 gm. substance: 0.2752 gm. CO₂ and 0.1070 gm. H₂O.

C₈H₁₈O. Calculated. C 73.85, H 13.85, M_D²⁰ 40.67.

Found. " 73.86, " 13.77, " 40.55.

2-Ethyl-Hexyl Iodide.—This iodide was prepared by heating the alcohol (50 gm.) with 1.1 equivalents of iodine and an excess of

red phosphorus at 180°C. for 1 hour. The product was washed, dried, and distilled. The yield was 80 per cent of the theoretical. The iodide boiled at 80–90°C. at 11 mm.

$$D_4^{20} = 1.3365$$

0.2234 gm. substance: 0.2170 gm. Ag I (Carius).

$C_8H_{17}I$. Calculated. I 52.92.

Found. " 52.48.

3-Methyl-Heptane.—The iodide was reduced in glacial acetic acid with zinc dust. The solution was saturated with hydrogen chloride and heated on the water bath for 3 days with frequent introduction of small portions of zinc. The hydrocarbon was then distilled in a current of steam collected in ether, and distilled with a fractionating column. It boiled at 120–122°C. at 755 mm. Clarke⁵ found 117.6°C. The yield was 84 per cent of the theoretical.

$$D_4^{20} = 0.7069$$

$$N_D^{20} = 1.3980$$

0.0965 gm. substance: 0.2994 gm. CO_2 and 0.1356 gm. H_2O .

C_8H_{18} . Calculated. C 84.21, H 15.79, M_D^{20} 39.14.

Found. " 84.59, " 15.72, " 38.92.

Diethyl 1-Methyl-Heptyl-Malonate.—1-Methyl-heptyl iodide was condensed with malonic ester (1.25 equivalents) in the usual manner. The yield was 80 per cent of the theoretical. The ester boiled at 157–158°C. at 10 mm.

$$D_4^{20} = 0.9496$$

$$N_D^{20} = 1.4324$$

0.1016 gm. substance: 0.2472 gm. CO_2 and 0.0928 gm. H_2O .

$C_{18}H_{36}O_4$. Calculated. C 66.18, H 10.29, M_D^{20} 74.78.

Found. " 66.34, " 10.22, " 74.36.

1-Methyl-Heptyl-Malonic Acid.—The ester was saponified with a large excess of sodium hydroxide. The acid was liberated from the sodium salt with dilute hydrochloric acid. Since it could not be made to crystallize, it was collected in ether, dried, and the ether removed by distillation. The residue was converted into the corresponding substituted acetic acid.

⁵ Clarke, L., *J. Am. Chem. Soc.*, 1911, xxxiii, 521.

3-Methyl-Nonylic Acid.—Crude 1-methyl-heptyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved. The residue was then distilled. It boiled at 147–148°C. at 12 mm.

$$D_4^{20} = 0.9012$$

$$N_D^{20} = 1.4342$$

0.0998 gm. substance: 0.2546 gm. CO₂ and 0.1036 gm. H₂O.

C₁₀H₁₈O₂. Calculated. C 69.77, H 11.63, M_D²⁰ 49.92.

Found. " 69.57, " 11.61, " 49.72.

Ethyl 3-Methyl-Nonylate.—The acid was esterified in the usual manner with alcohol and sulfuric acid. It boiled at 115°C. at 13 mm.

$$D_4^{20} = 0.8653$$

$$N_D^{20} = 1.4240$$

0.0982 gm. substance: 0.2598 gm. CO₂ and 0.1080 gm. H₂O.

C₁₂H₂₄O₂. Calculated. C 72.00, H 12.00, M_D²⁰ 59.27.

Found. " 72.15, " 12.19, " 58.98.

3-Methyl-Nonanol.—This alcohol was obtained by the reduction with sodium and alcohol of the corresponding ester. The yield was over 90 per cent. It boiled at 103–103.5°C. at 9 mm.

$$D_4^{20} = 0.8342$$

$$N_D^{20} = 1.4361$$

0.1014 gm. substance: 0.2826 gm. CO₂ and 0.1296 gm. H₂O.

C₁₀H₂₂O. Calculated. C 75.94, H 13.92, M_D²⁰ 49.91.

Found. " 76.00, " 14.29, " 49.53.

3-Methyl-Nonyl Iodide.—3-Methyl-nonanol was heated for 1 hour at 180°C. with 1.1 equivalents of iodine and an excess of red phosphorus. The iodide was then washed, dried, and distilled. It boiled at 115°C. at 10 mm. The yield was 90 per cent of the theoretical.

$$D_4^{20} = 1.2515$$

0.2430 gm. substance: 0.2130 gm. AgI (Carius).

C₁₀H₂₁I. Calculated. I 47.39.

Found. " 47.36.

3-Methyl-Nonane.—This hydrocarbon was prepared in the same manner as the 3-methyl-heptane. It distilled at 165–166.5°C. at 751 mm.

$$D_4^{20} = 0.7354$$

$$N_D^{20} = 1.4126$$

0.1012 gm. substance: 0.3140 gm. CO₂ and 0.1420 gm. H₂O.

C₁₀H₂₂. Calculated. C 84.51, H 15.49, M_D²⁰ 48.38.

Found. " 84.60, " 15.70, " 48.12.

2-Butyl-Hexanol.—2 Butyl-hexanol was prepared exactly as described by Levene and Cretcher.² It boiled at 210–212°C. at 731 mm.

2-Butyl-Hexyl Iodide.—The alcohol was heated at 180°C. for 1 hour with iodine and red phosphorus, washed, dried, and distilled. The iodide boiled at 115–117°C. at 8 mm. Levene and Crechter² found a boiling point of 124–125°C. at 13 mm.

0.2070 gm. substance: 0.1830 gm. AgI (Carius).

$C_{10}H_{21}I$. Calculated. I 47.39.
Found. " 47.76.

5-Methyl-Nonane.—2-Butyl-hexyl iodide was reduced with zinc dust in glacial acetic acid. The process has already been described. The hydrocarbon boiled at 164–166°C. at 755 mm. The boiling point found by Levene and Cretcher² was 165°C. The yield was 80 per cent of the theoretical.

$D_4^{20} = 0.7319$

$n_D^{20} = 1.4116$

0.1116 gm. substance: 0.3452 gm. CO_2 and 0.1574 gm. H_2O .

$C_{10}H_{22}$. Calculated. C 84.51, H 15.49, M_D^{20} 48.38.
Found. " 84.35, " 15.78, " 48.24.

4-Butyl-Octanol.—The procedure described by Levene and Cretcher² was followed for the conversion of 2-butyl-hexyl iodide into 4-butyl-octanol. The alcohol distilled completely at 127–130°C. at 8 mm.

4-Butyl-Octyl Iodide.—This iodide was prepared by heating the alcohol with iodine and red phosphorus at 180°C. It boiled at 145–146°C. at 10 mm. Levene and Cretcher² report 143°C. at 8 mm. The yield was 92 per cent of the theoretical.

0.2096 gm. substance: 0.1663 gm. AgI (Carius).

$C_{12}H_{25}I$. Calculated. I 42.91.
Found. " 42.87.

5-Propyl-Nonane.—4-Butyl-octyl iodide was reduced with zinc dust in glacial acetic acid. The method has already been described. The 5-propyl-nonane boiled at 204–205°C. at 763 mm. The yield was 75 per cent of the theoretical.

$$D_4^n = 0.7559$$

$$N_D^{20} = 1.4228$$

0.1004 gm. substance: 0.3112 gm. CO₂ and 0.1382 gm. H₂O.

C₁₂H₂₂. Calculated. C 84.71, H 15.29, M_D²⁰ 57.62.

Found. " 84.52, " 15.40, " 57.25.

Diethyl Butyl-Malonate.—This ester was prepared by the condensation of malonic ester and butyl iodide in the usual manner. It boiled at 122°C. at 12mm. Adams and Marvel⁶ found a boiling point of 144–145°C. at 40 mm. The yield was 75 per cent of the theoretical.

$$D_4^n = 0.9745$$

$$N_D^{20} = 1.4222$$

0.0988 gm. substance: 0.2209 gm. CO₂ and 0.0842 gm. H₂O.

C₁₁H₂₀O₄. Calculated. C 61.11, H 9.26, M_D²⁰ 56.31.

Found. " 60.97, " 9.53, " 56.35.

Diethyl Butyl-Heptyl-Malonate.—In the usual manner, diethyl butyl-malonate (1 equivalent) was condensed with heptyl iodide (1.1 equivalents). The new ester boiled at 177–178°C. at 12 mm. The yield was 90 per cent of the theoretical.

$$D_4^n = 0.9318$$

$$N_D^{20} = 1.4366$$

0.1002 gm. substance: 0.2521 gm. CO₂ and 0.1014 gm. H₂O.

C₁₈H₃₄O₄. Calculated. C 68.79, H 10.83, M_D²⁰ 88.63.

Found. " 68.61, " 11.32, " 88.22.

Butyl-Heptyl-Malonic Acid.—The ester was saponified by heating its alcoholic solution over night with a large excess of sodium hydroxide. The acid crystallized from dilute acetone in fine needles which melted at 117°C.

0.1014 gm. substance: 0.2420 gm. CO₂ and 0.0924 gm. H₂O.

C₁₄H₂₆O₄. Calculated. C 65.12, H 10.08,

Found. " 65.08, " 10.19,

2-Butyl-Nonylic Acid.—Butyl-heptyl-malonic acid was heated at 180°C. as long as carbon dioxide was evolved and the residue distilled. 2-butyl-nonylic acid boiled at 179°C. at 13 mm.

⁶ Adams, R., and Marvel, C. S., *J. Am. Chem. Soc.*, 1920, xlii, 310.

$$D_4^{20} = 0.8860$$

$$N_D^{20} = 1.4403$$

0.0993 gm. substance: 0.2661 gm. CO₂ and 0.1098 gm. H₂O.

C₁₂H₂₄O₂. Calculated. C 72.90, H 12.15, M_D²⁰ 63.77.

Found. " 73.08, " 12.37, " 63.69.

Ethyl 2-Butyl-Nonylate.—The acid was esterified by boiling its alcohol solution with sulfuric acid. The ester distilled at 115°C. at 1 mm.

$$D_4^{20} = 0.8560$$

$$N_D^{20} = 1.4290$$

0.0980 gm. substance: 0.2674 gm. CO₂ and 0.1088 gm. H₂O.

C₁₄H₃₀O₂. Calculated. C 74.38, H 12.38, M_D²⁰ 73.12.

Found. " 74.40, " 12.42, " 72.89.

2-Butyl-Nonanol.—The ester was reduced in the usual manner. The yield of alcohol was 72 per cent of the theoretical. Practically all the remainder was recovered as 2-butyl-nonylic acid. The alcohol boiled at 112–114°C. at 0.5 mm.

$$D_4^{20} = 0.8359$$

$$N_D^{20} = 1.4430$$

0.1013 gm. substance: 0.2899 gm. CO₂ and 0.1276 gm. H₂O.

C₁₃H₂₈O. Calculated. C 78.00, H 14.00, M_D²⁰ 63.76.

Found. " 78.06, " 14.09, " 63.43.

2-Butyl-Nonyl Iodide.—2-Butyl-nonanol was heated for 1 hour at 180°C. with iodine and red phosphorus. The product was washed with sodium thiosulfate, dried, and distilled. It boiled at 121–123°C. at 0.5 mm.

0.2116 gm. substance: 0.1602 gm. AgI (Carius).

C₁₃H₂₇I. Calculated. I 40.97.

Found. " 40.90.

5-Methyl-Dodecane.—This hydrocarbon was prepared by the reduction of the 2-butyl-nonyl iodide with zinc dust in glacial acetic acid. It boiled at 225.5–227°C. at 758 mm. The yield was 91 per cent of the theoretical.

$$D_4^{20} = 0.7576$$

$$N_D^{20} = 1.4244$$

0.1084 gm. substance: 0.3354 gm. CO₂ and 0.1516 gm. H₂O.

C₁₃H₂₈. Calculated. C 84.78, H 15.22, M_D²⁰ 62.23.

Found. " 84.37, " 15.64, " 62.02.

Oxidation of 5-Methyl-Nonane.—The procedure for the oxidation of the hydrocarbon was developed from that described by Levene and Cretcher.² The material (5 gm.) was shaken with 1,500 cc. of a solution containing 22.5 gm. each of potassium permanganate and potassium hydroxide at 37°C. The shaking was continued for 16 hours. Hydrogen peroxide was then added until the excess of permanganate was decomposed and the precipitated manganese dioxide filtered off. The volatile fatty acids were separated from the filtrate by acidifying and distilling in a current of steam as long as the distillate was acid to litmus. The acids were neutralized with sodium hydroxide and concentrated under diminished pressure to a volume of about 15 cc.

Since a preliminary experiment showed that the mixture of acids obtained could not be easily separated by crystallization of the silver salts, the procedure recommended by Crowell⁷ for the separation of butyric and acetic acids was tested in the following manner: 1 gm. of a mixture of 2 parts of butyric and 1 part of acetic acids was diluted to 15 cc., acidified with concentrated hydrochloric acid, and washed into a separatory funnel with two 25 cc. portions of saturated calcium chloride solution, containing 20 gm. of potassium chloride per liter, and two 25 cc. portions of kerosene. After shaking, the layers were separated and the kerosene was washed with 10 cc. of fresh calcium chloride solution. The kerosene was then extracted with sodium hydroxide, the alkaline solution acidified, and extracted with ether. The ethereal solution was treated with a few drops of ammonium hydroxide and allowed to evaporate over night. The residue should contain the ammonium salt of the butyric acid.

The calcium chloride solution was also extracted with ether and the ammonium salt prepared in the same manner. The residue should contain ammonium acetate.

These ammonium salts were washed separately into flasks and treated with saturated silver nitrate solution. The precipitates formed were crystallized from small amounts of water and analyzed.

Butyrate fraction:

0.1422 gm. substance on ignition: 0.0790 gm. Ag.

$C_4H_7O_2Ag$.	Calculated.	Ag 55.4.
	Found.	" 55.6.

⁷ Crowell, R. D., *J. Am. Chem. Soc.*, 1918, xl, 453.

Acetate fraction:

0.2216 gm. substance on ignition: 0.1370 gm. Ag.

$C_3H_7O_2Ag$.	Calculated.	Ag 64.7.
	Found.	" 61.8.

It is apparent that butyric acid can be obtained in a pure state from such a mixture but that acetic acid will be contaminated by a comparatively large proportion of the higher fatty acid.

The mixture of salts of the fatty acids obtained from the oxidation of 20 gm. of 5-methyl-nonane was therefore concentrated to about 15 cc. and fractionated by means of the kerosene extraction in the presence of the saturated calcium chloride solution. There were obtained 1.5 gm. of silver salt from the butyrate fraction and 1.4 gm. from the acetate fraction.

The butyrate fraction was crystallized from water and, after filtering, alcohol added to the mother liquor. The top fraction (I) weighed 1.3 gm. and the lower fraction (II), 0.1265 gm. They were both analyzed.

Fraction I. 0.1014 gm. substance on ignition: 0.0559 gm. Ag.

" II. 0.1265 " " " " 0.0699 " "

$C_4H_7O_2Ag$.	Calculated.	Ag 55.4.
	Found, Fraction I.	" 55.1.
	" " II.	" 55.3.

Fraction I was dissolved in dilute sulfuric acid, the fatty acid extracted with ether, and the ammonium salt prepared as before. The residue was treated with one-half the calculated quantity of silver nitrate and the precipitate (Fraction Ia, 0.2 gm.) filtered off. A second fraction was obtained from the filtrate by the addition of an excess of silver nitrate (Fraction Ib, 0.0519 gm.).

Fraction Ia. 0.1001 gm. substance on ignition: 0.0556 gm. Ag.

" Ib. 0.0519 " " " " 0.0283 " "

$C_4H_7O_2Ag$.	Calculated.	Ag 55.4.
	Found, Fraction Ia.	" 55.5.
	" " Ib.	" 54.5.

The butyrate fraction was therefore practically pure silver butyrate.

The acetate fraction was also crystallized from water. When the solution was heated there was marked blackening, indicating the presence of silver formate. Five fractions were obtained:

Fraction I, 0.6 gm.; Fraction II, 0.3 gm.; Fraction III, 0.2 gm.; Fraction IV, 0.0104 gm.; and Fraction V, 0.3 gm.

They were all analyzed:

Fraction	I.	0.1043 gm.	substance on ignition:	0.0639 gm.	Ag.
"	II.	0.1008 "	"	"	" 0.0597 "
"	III.	0.1001 "	"	"	" 0.0583 "
"	IV.	0.0104 "	"	"	" 0.0063 "
"	V.	0.1007 "	"	"	" 0.0633 "

$C_3H_5O_2Ag$.	Calculated.	Ag	64.7.
$C_3H_5O_2Ag$.	"	"	59.7.
$C_4H_7O_2Ag$.	"	"	55.4.
Found, Fraction	I.	"	61.3.
"	II.	"	59.2.
"	III.	"	58.2.
"	IV.	"	60.5.
"	V.	"	62.9.

In order to determine the amount of destruction of the fatty acids by permanganate, both acetic and butyric acids were separately subjected to the conditions of the oxidation of the hydrocarbon. Acetic acid was not attacked, since there was no loss of permanganate in 24 hours. The butyric acid was slowly oxidized.

1 gm. of butyric acid was shaken with 1,500 cc. of alkaline permanganate for 16 hours at 37°C. and the fatty acids were produced, isolated as before.

The butyrate fraction weighed 0.15 gm.

0.1004 gm. substance on ignition: 0.0553 gm. Ag.

$C_4H_7O_2Ag$.	Calculated.	Ag	55.4.
Found.	"	"	55.1.

From the acetate fraction 0.4 gm. were recovered: Fraction I, 0.3 gm.; and II, 0.1 gm.

Fraction I. 0.1004 gm. substance on ignition: 0.0621 gm. Ag.

" II. 0.1047 " " " " 0.0605 " "

$C_3H_5O_2Ag$.	Calculated.	Ag	64.7.
Found, Fraction	I.	"	61.9.
"	II.	"	57.8.

CERTAIN TRIPHENYLMETHANE DYES.*

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(Received for publication, August 5, 1922.)

The preparation in a pure state of a number of dyes of the malachite green series was undertaken as a part of a study of the bactericidal action of dyes in general. In the course of these studies we have had occasion to prepare a number of new members of this group and to make new observations on others. Although this work has been discontinued while still incomplete, we wish to present in brief tabular form the results of the chemical work as far as it has been carried. In a number of cases where the chlorides and sulfates proved too soluble for convenient manipulation, the nitrates were found to be of service because of their more sparing solubility and greater tendency to crystallize. Such substances as malachite green and brilliant green may be readily isolated and purified in this form. This is also true of the furfural analog of malachite green recently described as the zinc double chloride, the oxalate and the chloroplatinate by Renshaw.¹

The dyes were prepared in the usual way by oxidation of the leuco compounds with lead peroxide. The leuco compounds, in turn, were prepared either from the corresponding aldehyde and dialkyl aniline, or from the benzohydrol and substituted aniline.

* Presented at the Annual Meeting of the American Chemical Society, September, 1921.

¹ Renshaw, THIS JOURNAL, 44, 864 (1922).

TABLE I.
Leuco Compounds.

Derivative of leuco malachite green.	Preparation.	Crystalline form.	Melting point. °C.	Formula.	Analyses.		Remarks.
					Calc. N %	Found N %	
<i>m</i> -Acetamino-	From the -NH ₂ deriv. with acetic anhydride.	Faintly greenish rhombs from 85% alcohol.	154.5-155.5	C ₂₄ H ₂₇ ON ₃	10.85	11.14	Crystallized with difficulty.
<i>m</i> -Uramino-	From the -NH ₂ deriv. with KNCO in acetic acid.	Delicate needles from alco- hol.	194-196	C ₂₄ H ₂₇ ON ₄	14.43	14.43	
<i>p</i> -Diethyl-amino-	From Michler's hydrol and diethyl-aniline.	Cream-colored needles from benzene-alcohol.	142-144.5	C ₂₇ H ₃₃ N ₃	10.47	10.63	
<i>o</i> -Chloro- <i>p</i> - dimethyl-amino-	From Michler's hydrol and <i>m</i> -chloro-dimethyl- aniline.	Flat, tan needles from tol- uene-ligroin.	170-171.5	C ₂₄ H ₃₀ N ₃ Cl	10.31	10.55	
2-Hydroxy-5-phe- nylazo-	From the <i>o</i> -OH comp. and diazotized aniline in alka- line solution.	Lenticular platelets from benzene.	186-187	C ₂₄ H ₂₆ ON ₄	12.44	12.62	Did not yield a crystalline dye on oxidation.
2-Hydroxy-5-(<i>p</i> - methoxy-phe- nylazo)-	Using diazotized <i>p</i> -anisi- dine.	Ochreous prisms from ben- zene.	187-188	C ₂₆ H ₃₂ O ₂ N ₄	11.67	11.79	Purified with diffi- culty. Did not yield a crystal- line dye when oxidized.

TABLE II.
Dyes.

Derivative of malachite green.	Salt.	Crystal form.	H ₂ O of crist.	Decompos. pt. (anhydrous) °C.	Formula	Analyses.					Color with conc. H ₂ SO ₄ .
						Calc. N.		Found N.			
						H ₂ O %	(an- hydr.) %	H ₂ O %	(an- hydr.) %	Carbinol.	
Unsubstituted	nitrate.	olive platelets.	1 mol.	130-135	C ₂₂ H ₂₀ O ₃ N ₄ .H ₂ O	4.4	10.73	4.48	10.42	minute crysts.	orange.
<i>p</i> -Methyl-	chloride.	blue green leaf- lets.	4	160	C ₂₄ H ₂₇ N ₄ Cl.4H ₂ O	15.98	7.40	16.30	7.84	needles.	orange.
<i>o</i> -Chloro-	Cl ⁻	voluminous threads.	...	Intumesces 170° with preliminary softening.	C ₂₂ H ₂₄ N ₄ Cl ₂	...	7.02	...	6.80	octahedra.	orange.
<i>p</i> -Chloro-	NO ₃ ⁻	greenhairs, golden luster.	3.5	m. 120-175	C ₂₂ H ₂₀ O ₃ N ₄ Cl ₃ .3½H ₂ O	12.90	9.88	13.06	10.17	minute cryst.	bright orange.
<i>p</i> -Nitro-	Cl ⁻	purple needles.	5.5	150-155	C ₂₂ H ₁₈ O ₃ N ₄ Cl ₃ .5½H ₂ O	19.48	10.25	19.14	10.15	minute platelets.	bright orange.
<i>p</i> -Acetyl- methylamino-	NO ₃ ⁻	green needles, metallic lus- ter.	0.5 to 1	193-196	C ₂₂ H ₂₀ O ₄ N ₄ .H ₂ O	3.75	12.11	2.70	12.35	prisms.	orange.
<i>o</i> -Chloro- <i>p</i> - dimethyl- amino-	Cl ⁻	olive needles.	3.5	185-190	C ₂₂ H ₂₂ N ₄ Cl ₃ .3½H ₂ O	12.48	9.51	11.96	9.92	rhombs.	bright orange.
<i>m</i> -Uramino-	NO ₃ ⁻	olive-green needles.	4	185	C ₂₄ H ₂₇ O ₃ N ₄ .4H ₂ O	13.82	15.58	13.53	15.39	thin plate- lets.	bright orange.

TABLE II.—*Concluded.*

Derivative of malachite green.	Salt.	Crystal form.	H ₂ O of cryst.	Decompn. pt. (anhydrous) °C.	Formula.	Analyses.					Color with conc. H ₂ SO ₄ .
						Calc. N.		Found N.			
						H ₂ O %	(an- hydr.) %	H ₂ O %	(an- hydr.) %	Carbinol.	
<i>o</i> -Hydroxy-	Cl ⁻	bronze-dia- mond plate- lets.	0	195 with pre- liminary softening	C ₂₂ H ₂₅ ON ₃ Cl	...	7.36	...	7.16	rhombs.	dull greenish- blue.
<i>p</i> -Hydroxy-	Cl ⁻	hairs.	...	185-190	C ₂₂ H ₂₅ ON ₃ Cl	...	7.36	...	7.20	reddish needles.	yellowish- orange.
<i>o</i> -Methoxy-	NO ₃ ⁻	bronze needles.	...	?	C ₂₄ H ₂₇ O ₃ N ₃ .H ₂ O	4.10	9.99	4.12	9.71	leaflets.	olive-brown.
<i>m</i> -Methoxy-	carbinol.	beveled hex- agonal plates.	...	147-149.5	C ₂₄ H ₂₉ O ₃ N ₃	...	7.45	...	7.60	...	brown, with olive reflex.
<i>p</i> -Methoxy-	Cl ⁻	blue leaflets.	...	125-140	C ₂₄ H ₂₇ ON ₃ Cl	...	7.10	...	7.16	needles.	orange.
<i>o</i> -Ethoxy-	carbinol.	needles.	...	178-180	C ₂₄ H ₂₉ O ₃ N ₃	...	7.18	...	7.44	...	blue.
<i>p</i> -Ethoxy-	Cl ⁻	needles, leaflets.	...	150 with pre- liminary softening	C ₂₄ H ₂₉ ON ₃ Cl	...	6.85	...	6.95	rhombs.	orange.
3, 4-Methylene- dioxy-	Cl ⁻	bronze hairs.	4	155-160	C ₂₄ H ₂₅ O ₃ N ₃ .Cl.4H ₂ O	14.98	6.86	14.13	7.01	micro- crystals.	wine-red.
<i>o</i> -Hydroxyace- tic-acid	ether anhy- dride.	grass-green, platelets.	0	170-175	C ₂₂ H ₂₅ O ₃ N ₃	...	6.97	...	7.18	...	wine-red with olive reflex.
Furo-	NO ₃ ⁻	olive-green hairs, bronze luster.	5	190	C ₂₇ H ₂₅ O ₄ N ₃ .5H ₂ O	19.10	11.02	18.93	11.02	...	brown-purple.
Brilliant green	NO ₃ ⁻	brassy aggre- gates.	1	140-210	C ₂₇ H ₂₅ O ₄ N ₃ .H ₂ O	3.87	9.40	3.66	9.52	...	orange-brown.

ON DIACETONE GLUCOSE.

By P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 30, 1922.)

In a previous paper¹ it was reported that diacetone glucose and monoacetone benzylidene glucose on treatment with phosphorus oxychloride yield two phosphoric esters, which seem to differ one from another by the allocation of the phosphoric acid radical.

It was mentioned in this publication that additional and, if possible, more direct evidence of the structure of diacetone glucose and of acetone benzylidene glucose is desirable before a final discussion of the differences in the two phosphoric esters.

Two views have been advanced regarding the structure of diacetone glucose. According to the original view of Irvine and Scott,² the respective positions of the substituting groups were 1, 2 and 3, 5. Later, Macdonald,³ in Irvine's laboratory, revised the theory, ascribing to the second substituting group, the position 5, 6. Karrer and Hurwitz,⁴ on the basis of very imperfect experimental evidence, came to the same conclusion. On the other hand, Irvine and Hogg⁵ have later reversed the conclusion of Macdonald and attributed to the second substituting group the position 3, 5. Thus, at present, the two views are still under discussion.

The present communication contains direct evidence in favor of the theory that in diacetone glucose the positions of the acetone radicals are on carbon atoms 1, 2 and 5, 6.

According to the theory of Irvine and Hogg⁵ the methylation of diacetone glucose should yield 6-methyl glucose. This on oxidation

¹ Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1922, liii, 431.

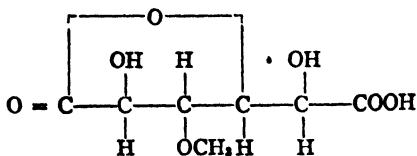
² Irvine, J. C., and Scott, J. P., *J. Chem. Soc.*, 1913, ciii, 568.

³ Macdonald, J. L. A., *J. Chem. Soc.*, 1913, ciii, 1896.

⁴ Karrer, P., and Hurwitz, O., *Helvetica Chim. Acta*, 1921, iv, 728.

⁵ Irvine, J. C., and Hogg, T. P., *J. Chem. Soc.*, 1914, cv, 1386.

should yield 6-methyl gluconic acid, whereas according to the other theory 3-methyl saccharic acid should be obtained. On oxidation of the methyl glucose prepared by us 1,4-anhydro-3-methyl saccharic acid of the following structure, was obtained.



The reasons which led Irvine and Hogg to a different conclusion undoubtedly lie in the fact that they failed to obtain the oxidation product of monomethyl glucose in crystalline form.

Work on the acetone benzylidene glucose is in progress.⁶

EXPERIMENTAL.

3-Methyl Glucose.—30 gm. of diacetone, which were prepared as previously described, were methylated with an excess of dimethyl sulfate by the method of Haworth,⁷ using 150 cc. of 30 per cent sodium hydroxide and 90 cc. of dimethyl sulfate. The temperature of the bath was kept at 70°C. The product of methylation was extracted with ether, the ethereal solution dried with anhydrous sodium sulfate, and the ether removed under diminished pressure. The colorless syrup was converted into the free sugar. For this purpose it was dissolved (25 gm.) in 400 cc. of 50 per cent alcohol containing 1.6 gm. of HCl, and heated in boiling water with reflux for 70 minutes. The acid was removed with silver carbonate, and the excess of silver with H₂S. The filtrate was decolorized with charcoal and concentrated to a syrup under diminished pressure without heat. The syrup was taken up in a small amount of dry methyl alcohol and allowed to crystallize in an ice-alcohol cooling mixture. The crude

⁶ At the proof-reading of this communication, the latest publication of Irvine and Patterson (Irvine, J. C., and Patterson, J., *J. Chem. Soc.*, 1922, cxxi, 2146) has reached us. In that publication they attribute to the diacetone glucose the structure of a γ -glucose. If that contention is further corroborated, the position 4 and not 3 will have to be assigned to the methyl group.

⁷ Haworth, W. N., *J. Chem. Soc.*, 1915, cvii, 8.

product was recrystallized several times from methyl alcohol. The substance was identical with that described by Irvine and Scott.² It melted at 158°. It had the following final optical rotation in water:

$$[\alpha]_D^{20} = \frac{+1.14^\circ \times 100}{1 \times 2} = +57^\circ$$

The substance analyzed as follows:

0.1030 gm. substance: 0.1638 gm. CO₂ and 0.0684 gm. H₂O.
 C₇H₁₁O₆. Calculated. C 43.30, H 7.22.
 Found. " 43.36, " 7.43.

1,4-Anhydro-3-Methyl Saccharic Acid.—2 gm. of methyl glucose were dissolved in 50 cc. of 50 per cent nitric acid and allowed to stand at room temperature for 42 hours. The solution was then divided onto two clock-glasses and rapidly evaporated on a boiling water bath to complete dryness. This was moistened with 5 cc. of 50 per cent nitric acid and again evaporated to dryness. The residue was then evaporated twice with 5 cc. of water.

The dry material was taken up in acetone and ether from which it was obtained crystalline. It sintered and turned dark at 190° and melted at 206-207°C.

The substance had the following optical rotation:

$$[\alpha]_D^{20} = \frac{+0.15^\circ \times 100}{0.5 \times 2} = +15^\circ$$

The substance analyzed as follows:

0.1077 gm. substance: 0.1612 gm. CO₂ and 0.0490 gm. H₂O.
 C₇H₁₀O₇. Calculated. C 40.76, H 4.89.
 Found. " 40.81, " 5.09.

0.1006 gm. of substance dissolved in water required for immediate titration 5.4 cc. of 0.1 N alkali (phenolphthalein as indicator). On allowing this solution to stand for 1 hour with an excess of 0.1 N NaOH, 10.2 cc. of 0.1 N alkali were utilized. The theory for the lactone requires 4.85 cc. of 0.1 N alkali and for the dibasic acid 9.70 cc. of 0.1 N alkali.

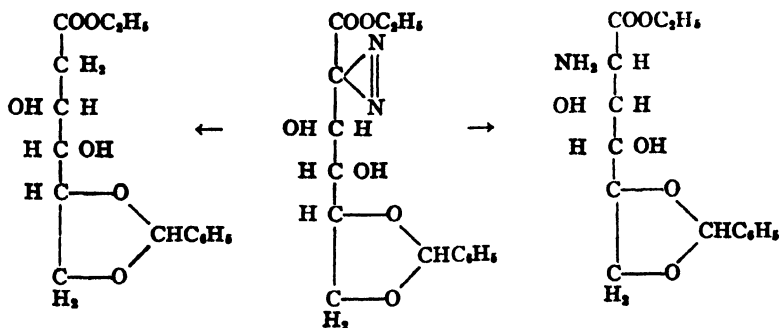
REDUCTION OF BENZYLIDENE-1-ETHYL-2-DIAZO-GLUCONATE.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, October 28, 1922.)

It was previously reported that benzylidene-1-ethyl-2-diazogluconate, on treatment with acetic acid, with dry hydrogen chloride, and with dry hydrogen bromide, is transformed, respectively, into the benzylidene esters of gluconic, chloromannonic, and bromomannonic acids.¹ In no instance could the epimers of these substances be detected. In the present communication are reported the reduction experiments with aluminum amalgam. Two products are obtained as a result of this reaction; one is benzylidene-ethyl-chitosamine and the other 5,6-benzylidene-1-ethyl-2-desoxygluconate (mannonate). The formation of the chitosamine derivative took place apparently asymmetrically, as the crude product, judging from its optical rotation, contained no traces of its epimer. The reaction proceeded in the following way:



The desoxygluconic derivative was found identical with the product obtained earlier by the reduction of 5,6-benzylidene-1-ethyl-2, 3-

¹ Levene, P. A., *J. Biol. Chem.*, 1922, liii, 449.

* The substance should be graphically presented in this form and not as on page 452, Vol. liii of this *Journal*. The error was overlooked in proof-reading.

renewals of the amalgam were required to complete the reduction. Evolution of ammonia gas was noted soon after the beginning of the reaction. After the reduction was completed the clear filtrate from the aluminum was concentrated under diminished pressure, and the residue, at first syrupy, on standing turned into a crystalline mass. This was transferred into an evaporating dish by means of warm ether in which the residue dissolved readily. On standing it soon crystallized in long prismatic needles. The yield of crystalline material was 1.0 gm. for each 10 gm. of the original material. From the mother liquor further crops could be obtained. This, however, was not done, so as not to affect the maximum yield of the chitosaminic acid derivative contained in the filtrate.

The crystals were found insoluble in water and in dilute aqueous mineral acids, but very soluble in ether, alcohol, methyl alcohol, and acetone. For analysis it was recrystallized out of ether. It melted at 124°C. (corrected) and analyzed as follows:

0.1014 gm. substance: 0.2272 gm. CO₂ and 0.0620 gm. H₂O.
 C₁₅H₂₀O₆. Calculated. C 60.81, H 6.76.
 Found. " 61.10, " 6.84.

The rotation of the substance was:

$$[\alpha]_D^{25} = \frac{-0.56 \times 100}{1 \times 2} = -28.0^\circ$$

Thus this substance was identical with the benzylidene-1-ethyl-2-desoxygluconate described in a previous publication. The mother liquor contained the derivative of chitosaminic acid. The original mother liquor was taken up in 100 cc. of 2 per cent sulfuric acid and placed on a water bath until all the ether was evaporated and all the benzaldehyde settled out on the bottom of the flask as a yellow oil. The reaction product was cooled, the benzaldehyde extracted with ether, and the remaining aqueous layer allowed to stand over night with a slight excess of baryta water. The excess of barium was then removed quantitatively with sulfuric acid. The combined mother liquors of two experiments of 10.0 gm. each, were analyzed for amino nitrogen and showed after 4 minutes shaking 0.090 gm. of nitrogen, equivalent to 1.26 gm. of chitosaminic acid. This aqueous solution

was concentrated under diminished pressure to a thick syrup, which was transferred to a small beaker by means of a little methyl alcohol. To the solution methyl alcohol was added until a small sticky precipitate appeared. The beaker was placed on a water bath and the contents were stirred until the chitosaminic acid began to crystallize. The yield of the crystalline material was 0.60 gm. This material without further purification had the optical rotation: $[\alpha]_D^{20} = -15.0^\circ$ thus showing that it consisted only of chitosaminic acid without any admixture of its epimer. For purification the combined material obtained from 60 gm. diazo compound was recrystallized twice from dilute methyl alcohol. The final substance analyzed as follows:

0.0200 gm. were dissolved in 5 cc. of water.

2 cc. of this solution: (Van Slyke) 1.07 cc. N_2 at $26^\circ C.$, 757.4 mm.

$C_8H_{12}NO_6$. Calculated. N 7.18.

Found. " 7.26.

Its optical rotation was:

$$[\alpha]_D^{20} = \frac{-0.15^\circ \times 100}{1 \times 1} = -15.0^\circ$$

In order to test the purity of the diazo derivative the substance (20.0 gm.) was hydrolyzed with dilute sulfuric acid and treated in exactly the same manner as described above. The resulting solution showed the presence of 0.003 gm. of nitrogen, practically a negligible quantity.

Reduction of Benzylidene-1-Amino-2, 3-Anhydrogluconate (Mannonate).—The substance (5.0 gm.) was dissolved in 300 cc. of isopropyl alcohol and freshly prepared aluminum amalgam was added (1.0 gm.). Only a slight evolution of gas was noted. After 2 hours the reaction product was filtered and to the filtrate another portion of aluminum amalgam was added. The flask was allowed to stand overnight, and the clear filtrate concentrated. The residue crystallized in the distilling flask. The residue was dissolved in hot ether and transferred to a crystallizing dish. The substance crystallized very soon and was filtered off after several hours. The mother liquor on evaporation

gave a very insignificant residue. The crystalline material melted at 230°C. and analyzed as follows:

0.1068 gm. substance: 0.2308 gm. CO₂ and 0.0564 gm. H₂O.

C₁₃H₁₃NO₃. Calculated. C 58.84, H 5.70.
 Found. " 58.90, " 5.90.

The optical rotation was:

$$[\alpha]_D^{25} = \frac{-1.32^\circ \times 100}{1 \times 2} = -66.0^\circ$$

HYDROLYSIS OF YEAST NUCLEIC ACID WITH DILUTE ALKALI AT ROOM TEMPERATURE. (CONDITIONS OF STEUDEL AND PEISER.)

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, November 22, 1922.)

In the year 1919, Steudel and Peiser¹ published a communication on the structure of yeast nucleic acid in which they challenged the hypothesis developed in this laboratory. The authors took exception to the tetranucleotide theory, offering in its place a trinucleotide theory. They claimed that the complex yeast nucleic acid was composed of guanine, adenine, and cytosine nucleotides, and further, that the uridine nucleotide present in the commercial product (C. F. Boeringer and Söhne) was an impurity of independent origin. In a later publication (1921) they reported² the discovery in commercial nucleic acid, of another impurity; namely, guanylic acid. Finally, in 1922³ the same authors stated that yeast nucleic acid on treatment with a 3 per cent solution of sodium hydroxide at 15-17°C. liberates from its molecule 1 molecule of guanylic acid, so that the remaining part is completely free from guanine. Steudel and Peiser considered their observation of such importance that they advocated for the present the deferring of the discussion of the structure of yeast nucleic acid.

To the present writer, on the contrary, the observation of Steudel and Peiser seemed to substantiate the theory of the structure of the yeast nucleic acid formulated by him, according to which the individual nucleotides are linked to one another in ester form. A complete analysis of the products formed under the conditions given by Steudel and Peiser confirms still further the theory expressed by

¹ Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1919, cviii, 42.

² Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1921, cxiv, 201.

³ Steudel, H., and Peiser, E., *Z. physiol. Chem.*, 1922, cxx, 292.

us, since it was found that simultaneously with the formation of guanylic acid, the other three nucleotides also are formed. Thus all doubts as to the tetranucleotide conception of yeast nucleic acid are removed. The statement of Steudel and Peiser that under their conditions the liberation of guanylic acid is complete was found to be erroneous. Three experiments were performed at three temperatures varying between 15 and 25°C. In each experiment a portion of the nucleic acid remained unchanged. At the highest temperature, 12 per cent, at about 20°C., 37 per cent, and at 15°C., 52 per cent remained undecomposed. No attempt was made to regulate the temperature accurately, the variations were caused by weather conditions. The details of the procedure for the separation of the unchanged nucleic acid from the simple nucleotides and for the separation of guanylic acid from the other three nucleotides are given in the experimental part.

EXPERIMENTAL.

Nucleic acid (100 gm.) was dissolved in 400 cc. of a 3.5 per cent solution of sodium hydroxide and allowed to stand at room temperature over night (15 hours). The solution was then separated into three fractions; the first contained unchanged nucleic acid, the second, guanylic acid, and the third, the other three nucleotides.

Nucleic Acid Fraction.—The separation of the unchanged nucleic acid was brought about by precipitation with glacial acetic acid. To test the efficiency of this procedure, purified nucleic acid (40.0 gm.) was dissolved in 130 cc. of water on addition of a little alkali and precipitated with 5 liters of glacial acetic acid. The precipitate was washed with alcohol and ether and dried under diminished pressure over sulfuric acid. The yield of the substance was 39.0 gm. or 97.5 per cent of the original material.

The composition of the two samples was as follows:

	C	H	N	P	Purine bases as hydro- chlorides.
					<i>per cent</i>
Original material (No. 101).....	34.79	4.19	15.70	9.05	36
Recovered material (No. 172).....	34.57	4.16	15.58	9.04	38

In the experiments on the action of dilute alkali, 400 cc. of the solution were poured into 20 liters of glacial acetic acid. A precipitate formed, which was dried as described above. The yield of the recovered nucleic acid varied with the room temperature. At 15°C. the yield was 52 per cent, at 20°C., 37 per cent, and at 25°C., 12 per cent. The composition of the recovered material was as follows:

	C	H	N	P	Purine bases as hydro- chlorides. per cent
From experiment at 15°C. (No. 104).....	34.17	4.15	15.62	8.65	39
From experiment at 20°C. (No. 153).....	34.31	4.04	15.86	8.84	37

Guanylic Acid Fraction.—The mother liquors from the first fraction were concentrated under diminished pressure (about 10 to 15 mm.) without heating to nearly dryness. The residue was dissolved in about 100 cc. of water (in experiments starting with 100 gm. of nucleic acid) and allowed to stand over night in the refrigerator at a temperature varying from 0 to + 2°C. The acid sodium salt of guanylic acid settled out in the form of a white precipitate and was readily filtered off by suction in the refrigerating chamber. The substance was converted into the lead salt, and this into the free acid. In order to remove all the base the operation had to be repeated three times. Finally, the solution which, on standing over night at 0 to + 2°C., remained perfectly clear, was converted into the brucine salt. After three recrystallizations from 35 per cent alcohol the substance analyzed as follows:

0.1017 gm. substance: 0.1984 gm. CO₂ and 0.0590 gm. H₂O.
 0.2000 " " 17.4 cc. N₂, *t* = 15°C., *P* = 769.5 mm.
 0.3000 " " 0.0274 gm. Mg₂P₂O₇.

Brucine salt of guanylic acid (air-dry).

$C_{26}H_{42}N_{10}O_{13} \cdot C_{10}H_{14}N_2 \cdot PO_3 + 7 H_2O$.

Calculated. C 52.61, H 6.28, N 9.88, P 2.43.

Found. " 53.02, " 6.48, " 10.09, " 2.81.

Fraction Containing Adenine, Cytosine, and Uridine Nucleotides.—The mother liquors from the guanylic acid fractions obtained from the three experiments were combined and converted into the lead

salts and these again freed from lead. It was found advisable to repeat this operation several times. The final solution of the nucleotides which reacted acid to Congo red was treated with a methyl alcoholic solution of brucine until the reaction of the original solution turned slightly alkaline. The brucine salts were then fractionated from 35 per cent alcohol exactly in the manner previously described. Three fractions were obtained differing from one another in their solubilities. The most insoluble portion had the properties and the composition of the brucine salt of uridine phosphoric acid. It analyzed as follows:

0.1011 gm. substance: 0.1966 gm. CO_2 and 0.0612 gm. H_2O .
 0.2000 " " 12.8 cc. N_2 , $t = 22^\circ\text{C}$., $P = 755.3$ mm.
 0.3000 " " 0.0272 gm. $\text{Mg}_3\text{P}_2\text{O}_7$.

Brucine salt of uridine phosphoric acid.

$\text{C}_{20}\text{H}_{28}\text{N}_4\text{O}_9 \cdot \text{C}_9\text{H}_{12}\text{N}_2 \cdot \text{PO}_3 + 7 \text{H}_2\text{O}$.

Calculated. C 53.30, H 6.43, N 6.79, P 2.50.

Found. " 53.02, " 6.77, " 7.43, " 2.53.

The intermediate fraction approached analytically the composition of cytidine phosphoric acid.

0.1011 gm. substance: 0.1594 gm. CO_2 and 0.0588 gm. H_2O .
 0.2000 " " 15.4 cc. N_2 , $t = 22^\circ\text{C}$., $P = 755.3$ mm.
 0.3000 " " 0.0272 gm. $\text{Mg}_3\text{P}_2\text{O}_7$.

Brucine salt of cytidine phosphoric acid.

$\text{C}_{20}\text{H}_{28}\text{N}_4\text{O}_9 \cdot \text{C}_9\text{H}_{14}\text{N}_2 \cdot \text{PO}_3 + 7 \text{H}_2\text{O}$.

Calculated. C 53.34, H 6.52, N 7.93, P 2.57.

Found. " 52.70, " 6.50, " 8.85, " 2.45.

Finally, the most soluble fraction had the composition of adenosine phosphoric acid.

0.1004 gm. substance: 0.1948 gm. CO_2 and 0.0590 gm. H_2O .
 0.2000 " " 17.0 cc. N_2 , $t = 24^\circ\text{C}$., $P = 766.6$ mm.
 0.3000 " " 0.0272 gm. $\text{Mg}_3\text{P}_2\text{O}_7$.

Brucine salt of adenosine phosphoric acid.

$\text{C}_{20}\text{H}_{28}\text{O}_9\text{N}_4 \cdot \text{C}_{10}\text{H}_{14}\text{N}_2 \cdot \text{PO}_7 + 7 \text{H}_2\text{O}$.

Calculated. C 53.28, H 6.40, N 10.00, P 2.47.

Found. " 52.92, " 6.57, " 9.86, " 2.53.

No attempt was made to isolate the individual nucleotides in the free state, since the isolation of the brucine salt is sufficient evidence to prove the contention that the bonds between the individual nucleotides of the yeast nucleic acids are very feeble. Thus the theory of an ester linking between the nucleotides is the one which best harmonizes with the experimental facts.

PURE CULTURES OF LARGE MONONUCLEAR LEUCOCYTES.

By ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 29 TO 37.

(Received for publication, March 17, 1922.)

I.

INTRODUCTION.

Pure strains of cells are as necessary in the study of physiological problems as pure cultures of microorganisms in bacteriology. If leucocytes and lymphocytes could be kept for a long time in active condition in media of known composition, many fundamental facts concerning their normal function in the organism and their reactions against bacteria and foreign material would be elucidated. In 1920, several attempts were made in this laboratory to obtain a pure culture of large mononuclear leucocytes. Finally, a strain was isolated from the blood of a hen in January, 1921, by one of us, and maintained in active condition for nearly 3 months. Since then, other strains have been cultivated in the same manner. The purpose of this paper is to describe the characteristics of the pure cultures, the morphological changes of the cells, and their response to certain modifications in the composition of the culture medium.

II.

Technique.

1. *Preparation of the Cells.*—The blood of an adult chicken which had fasted for 24 hours was taken in cold paraffined tubes through an oiled cannula or syringe and centrifuged at high speed for 10 minutes. After removal of the plasma, a few drops of diluted embryonic tissue juice were placed at the surface of the buffy coat of

leucocytes. 15 minutes later, the coagulum containing the white cells could be removed and placed in a watch-glass with a small amount of Ringer solution. Generally, the film separated into clumps from which almost all the red cells could be washed away. Small fragments were selected and taken from the fluid with the point of a cataract knife.

2. *Preparation of the Medium.*—Several different media were used. In some experiments, no embryonic tissue juice was used and the medium was composed of plasma alone, or of plasma and Tyrode solution. In other experiments, the medium consisted of two volumes of plasma and one volume of chick embryo juice, or of one volume of plasma, one volume of chick embryo juice, and two volumes of Tyrode solution. Sometimes 20 per cent fibrinogen suspension was substituted for the plasma. In order to prevent the contamination of the white cells by fibroblasts or other cells, the tissue juice was diluted with Tyrode solution and centrifuged for a long time. After the mixture of tissue juice and plasma had been made, the culture medium was examined microscopically and the presence of any contaminating cells could be detected easily. In other cases, the tissue extract, before being used, was kept in cold storage for several weeks until the contaminating cells were dead.

3. *Preparation of the Cultures.*—The culture medium was placed on a cover-glass of mica, and a small fragment of the leucocyte film was embedded in it before coagulation. A certain relation should exist between the number of white blood corpuscles and the volume of the medium. If the leucocytes were too few in number or if the medium was overcrowded by the cells, the result was negative. When the culture was properly prepared, a great many ameoboid cells were seen in the medium after 24 hours. Then the original fragment, which still contained a large number of cells, and the invaded area of the coagulum were divided into several parts and washed in Ringer solution. The section of the coagulum had to be made with greater care than in cultures of fibroblasts. If the knife was not very sharp and the edges of the coagulum were crushed or slightly folded, the cells could not migrate into the new medium. They multiplied in the old coagulum and died. After the first passage, the transfer into a fresh medium was made every 48 hours and even every 3rd, 4th,

or 5th day. The cultures were examined while living, and drawings and photographs were made of the cells. Some of them were stained with neutral red in concentrations varying from 1:10,000 to 1:40,000. A few cultures were fixed in warm Ringer solution containing 4 per cent of formalin, and stained with hematoxylin-eosin, Giemsa's stain, eosin and methylene blue, eosin-azure, and azure.

III.

RESULTS.

The first strain of large mononuclear leucocytes was kept in a condition of active life for almost 3 months. Some other strains were used for shorter periods of time.

1. Migration and Multiplication of the Cells.—After 24 hours, the fragment of coagulum containing the white blood corpuscles was surrounded by a very large number of cells which sometimes invaded the entire area of the medium (Fig. 1). The outer zone was made up of small ameboid cells. In fixed and stained cultures they appeared as polymorphonuclear leucocytes which are pseudo-eosinophilic. The inner zone consisted chiefly of larger ameboid cells. Some of them were very large and more or less elongated and branched, with reticular pseudopods. The others were smaller, more rounded in form, and emitted active filiform or lobar pseudopods. In the preparations stained with eosin-azure, these cells contained a darkly stained nucleus and some showed blue granules in their cytoplasm. They were lymphocytes and large mononuclear leucocytes. Some polymorphonuclear cells were also present in the inner zone. After 24 hours, fragments of coagulum containing the ameboid cells were extirpated from the cultures and placed in a new medium. The cells migrated almost immediately from the old into the new coagulum. They consisted of polymorphonuclear leucocytes, lymphocytes, and large mononuclears, but the number of polymorphonuclear leucocytes had markedly decreased (Fig. 2). If the cells were too closely packed in the old coagulum, their migration into the new medium was slow and death often occurred. After the third or fourth passage, no granular leucocytes remained in the cultures, which were composed merely of lymphocytes and large mononuclear cells. Later, the lymphocytes disappeared also (Fig. 3).

The activity of the cultures was irregular. The migration of the cells (Figs. 4 to 6) from the edges of the old coagulum started from the 1st to the 4th day. When a sharp section of the coagulum was made in the zone of the migrating cells, the medium was invaded sooner. During the first 24 hours, the cells multiplied in the old coagulum and accumulated in large numbers at the periphery where they sometimes formed masses of amorphous material. Later, a few ameboid cells grew from the edges (Figs. 4 to 6) and more or less suddenly toward the 3rd day a large number of them invaded the new medium. In other cases, the invasion of the medium began on the 1st day. The cells were usually disposed in chains which followed almost parallel paths into the new medium on several different planes, but every chain was laterally isolated from the others. When the growing masses were very dense and the cells in contact, the cultures generally died. Often chains of independent cells migrated far into the new medium. This phenomenon occurred generally after 2, 3, or 4 days, according to the technique used, the composition of the medium, and the previous activity of the cells. The multiplication of the cells was slow. However, a few mitotic figures were observed (Figs. 7 and 8). A normal culture was composed of a thick crown of elongated and branched cells, while the central part of the coagulum was practically free of cells. When the old coagulum contracted, or when the crushing or folding of its edges prevented migration in a measure, living cells and apparently dead material were observed in the center of the culture.

The general appearance of the cultures was strikingly different from that of a fibroblast culture. The cells had no tendency to form a tissue (Figs. 2 and 3), but always remained isolated. If they happened to be packed together, they died. When colonies of leucocytes were grown in Gabritschewski dishes, their edges showed no tendency to unite (Fig. 9).

The large mononuclear leucocytes invading the culture medium were almost uniform in shape, elongated or branched, and of irregular form. Their posterior end was generally rounded, while the anterior end emitted very active reticular pseudopods (Figs. 4 to 6). The nucleus was darkly stained by azure while the cytoplasm was clear. In the living cultures stained with neutral red, the cells showed a

segregation apparatus consisting of spherical bodies, often as large and even larger than the nucleus. After each passage, chains of cells of the same type, more or less densely packed, again invaded the medium. But under the influence of many technical conditions, and especially the composition of the culture medium, their rate of proliferation was liable to decrease. The two first strains which were isolated in January, 1921, lost their activity at the beginning of April and died soon afterwards. Another is still active after 3 months of life *in vitro*.

2. *Differentiation of the Large Mononuclear Leucocytes.*—In actively growing cultures, the appearance of the cells remained uniform. The strains were composed of ameboid cells, with more or less irregular darkly stained nuclei, and lobar, filiform, or reticular pseudopods (Figs. 5 and 6) which wandered from the edges of the old coagulum into the new medium. However, important morphological changes sometimes occurred. They were observed for the first time in December, 1920. Leucocytes had been taken from the blood of a hen and cultivated in homogenic plasma without the addition of any tissue juice. The coagulum was soon invaded by a large number of ameboid cells, which wandered through it for 2 days. Some of the cells agglutinated in clumps. At the periphery of, or within the clumps, a few cells modified their shape. The pseudopods disappeared and were replaced by sharp processes. On the 3rd day, fusiform and stellate cells resembling fibroblasts were seen in some of the clumps. There was no possible contamination of the culture by fibroblasts from another source because the cells had been taken from the blood and no tissue juice had been added to the plasma composing the medium.

The same phenomenon (Fig. 10 *a* and *b*) was observed subsequently in many cultures. It occurred generally when the migration and the multiplication of the lymphocytes were not very active. The transformation of the cells started in the old coagulum and its origin was multicentral. The cells which showed the first evidence of a change were the elongated lymphocytes with clear cytoplasm and reticular pseudopods. The central end of the cell lost its pseudopods and became a pointed and immobile process, while the peripheral end was still actively ameboid. These transition forms were apparently half fibroblasts and half ameboid cells. Sometimes the nucleus of an

ameboid cell lost its character of being darkly stained by azure, and became pale and oblong with two nucleoli like that of a typical fibroblast. Similar changes occurred in polygonal and branched cells which showed simultaneously fixed processes and reticular pseudopods. At the same time, typical macrophages (Fig. 11*b*) were seen at the periphery of the culture and sometimes in the immediate vicinity of the transition forms and the fibroblasts (Figs. 10*b* and 11*a*). The fibroblasts appeared often near the amorphous masses resulting from the accumulation of lymphocytes on the edges of the old coagulum. Pointed processes seemed to grow from this apparently necrotic material, and after a few hours, fusiform cells with slender processes could be seen. In a few cultures, the fibroblasts migrated from the old coagulum into the new medium where ameboid cells were still wandering (Fig. 10*b*). They were spindle-shaped or stellate cells with an outline not sharply defined, anastomotic processes, pale homogeneous cytoplasm, large oval nuclei, and generally two nucleoli (Fig. 12*a* to *d*). Of four cells united in a chain, two resembled spindle-shaped fibroblasts, the third was half fibroblast and half ameboid, and the fourth was a large mononuclear leucocyte. In this case it seemed that a chain of large mononuclears was being changed into fibroblasts, the mutation beginning in the central part of the coagulum. In other cultures, several fibroblasts were united by long processes in a reticulum.

Cultures which contained large mononuclears, transition forms, and cells resembling fibroblasts were stained with a weak solution of neutral red. The ameboid cells displayed large red granules, which sometimes filled the cytoplasm almost completely. Among the transition cells, some showed a large segregation apparatus and others small red granules. The fibroblastic forms presented very much finer granules, and some were completely free of them. In the latter case, the nucleus was oblong and clear, contained two nucleoli, and looked like the nucleus of a fibroblast. At the same time that their appearance was modified, the behavior of the cells changed. Instead of remaining isolated or forming a chain, they became able to unite sidewise with other cells and showed a tendency to form a tissue, while lymphocytes lived like isolated elements. The cells were acquiring not only a new morphological appearance but also the

property of associating with homologous cells in the manner of fibroblasts.

3. Action of Embryonic Tissue Juice and Serum on the Activity of the Cells.—It seemed probable that lymphocytes and large mononuclear leucocytes would respond to the presence of embryonic tissue juice and serum in the medium as do fibroblasts. But the sensitiveness of their reaction had to be investigated.

(a) In the experiments in which the action of embryonic tissue juice was tested, two kinds of media were used. The first was composed of 33.3 per cent normal plasma and 66.6 per cent of a mixture in different concentrations of embryonic tissue juice and Tyrode solution. The second was made of fibrinogen suspension, serum, Tyrode solution, and embryonic tissue juice. Large mononuclears in a condition of low activity at the third or fourth passage were cultivated in media containing 2.5, 33.3, and 66.6 per cent tissue juice. In 2.5 per cent tissue juice, very little or no migration took place and death occurred after one or two passages. On the contrary, the medium containing 33.3 per cent tissue juice was invaded by a large number of lymphocytes. In 66.6 per cent tissue juice, the migration was still greater, but the cells died after a short time, possibly because they were too closely packed. Similar experiments were made with a medium composed of 20 per cent fibrinogen suspension and 10 per cent serum, the presence of serum being necessary to obtain a firm clot which would not dissolve secondarily. The action of 2.5 and 40 per cent embryonic tissue juice was compared. The number of migrating cells was very much larger in the medium containing 40 per cent tissue juice, but they died sooner than in the 2.5 per cent medium. It was evident that the cells were very sensitive to the influence of the embryonic tissue juice, and that its optimum concentration in the medium was about 30 per cent.

(b) The action of homologous serum was studied in media composed of 20 per cent fibrinogen suspension, 5 per cent tissue extract, and varied concentrations of Tyrode solution and serum. The cells were obtained from cultures at the third and fourth passages and cultivated in media containing 10 and 50 per cent serum. In the first medium, no migration took place. The cells located in the old coagulum became round, and very few showed active ameboid

movements. They died after the first passage. On the contrary, in the second medium the cells located in the old coagulum retained their activity and a large number migrated into the new medium. After they were transferred to fresh medium, they perpetuated themselves for several generations. Similar results were obtained in media containing 70 and 10 per cent serum. There was no doubt that serum had a marked inhibiting influence on the migration of lymphocytes and large mononuclear leucocytes.

IV.

DISCUSSION.

The cells growing in pure cultures were large mononuclear leucocytes of Ehrlich (endothelial leucocytes, monocytes of Naegeli, blood histiocytes of Aschoff and Kiyono, or hemomacrophages of Metchnikoff). The pseudo-eosinophilic and eosinophilic leucocytes were never observed to be present after five or six passages. The lymphocytes, that is the small and medium sized mononuclear cells of Ehrlich, were seen in large numbers during the 1st week and disappeared afterwards spontaneously. Possibly they were transformed into larger forms, as Maximoff¹ observed in his cultures of lymphoid tissue. Then the cultures were composed exclusively of large mononuclear leucocytes. These cells maintained their activity *in vitro* for nearly 3 months. However, their rate of multiplication was slower than that of fibroblasts. The medium was probably not the optimum one. It may also be supposed that they cannot grow in solid medium in dense masses as fibroblasts do. When they were packed in the coagulum, they usually died after a short time. The actively growing cultures showed a large number of chains of ameboid cells radiating from the original fragment through the medium. But the chains were not in contact laterally. It seemed that the cells created around themselves a field unfavorable to homologous cells. They always remained isolated, while fibroblasts and epithelial cells in pure cultures formed a tissue. The colonies of lymphocytes and fibroblasts when in contact assumed a strikingly different appearance. The edges of fibroblast colonies had a marked tendency to unite.

¹ Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 225.

In some cultures, the fibroblasts of a colony seemed to be attracted by the fibroblasts of the neighboring colony and a group of several colonies soon formed a continuous layer of connective tissue (Fig. 13 *a*). On the contrary, the peripheral edges of the leucocyte colonies never coalesced, even when growing in close contact (Fig. 13 *b*).

The large lymphocytes were more sensitive to all influences than the fibroblasts. The slightest error in technique brought about the death of the cultures. This peculiarity increased the difficulties of the experiments very much, because marked differences of growth were observed which were due merely to technical errors. However, the cells responded readily to physical and chemical modifications of the composition of the medium by changes in the rate of migration and in their morphology. As cells cultivated *in vitro* respond to an antigen by the production of an antibody,² the use of pure strains of macrophages may, therefore, become of interest in the study of immunity.

The large mononuclears became transformed into cells which assumed the appearance of fibroblasts. These cells were spindle-shaped or stellate, with long and slender processes uniting sometimes in a reticulum with those of other cells. When stained vitally with neutral red, they could be distinguished from the large mononuclears present in the same culture by the neutral red reaction of Evans.³ The nucleus was similar to that of the fibroblasts. They had also acquired a tendency to tissue formation which lymphocytes do not possess. Besides the typical cells, there were many transition forms characterized by a nucleus less darkly stained and more oval, by the progressive substitution of sharp processes for the pseudopods, and by a decrease in the size of the segregation apparatus. The macrophages had evidently differentiated into fibroblasts. It is probable that Maximoff observed the beginning of a similar transformation in his cultures of lymphoid tissue.¹ He mentions that the large lymphocytes cultivated in hypotonic plasma and bone marrow extract underwent a modification. Their cytoplasm was larger and less basophilic, while the nucleus became paler.

² Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

³ Evans, H. M., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 3.

The differentiation of large mononuclears into fibroblasts is merely the confirmation of previous observations. Long ago, Renaut⁴ found that young connective tissue formed inside uterine cysts, completely lined with epithelial cells, and thought it derived from ameboid cells which had migrated through the epithelium. He considered that fixed connective tissue cells, endothelial cells, clasmotocytes, chromoblasts, vacuolar cells, and osteoblasts originated from an indifferent lymph cell. The transformation *in vitro* of connective tissue macrophages into fibroblasts came as a partial confirmation of this view. In 1912, pure cultures of ameboid cells from connective tissue became transformed into fibroblasts.⁵ Cultures of peritoneal macrophages were observed by Maximoff⁶ to undergo differentiation and to yield colonies of fibroblasts. Maximoff^{6,7} never observed the transformation of polyblasts into fibroblasts, probably for technical reasons. Foot,⁸ in cultivating white blood corpuscles of chicks, saw no real fibroblasts in the preparations. But in cultures of leucemic blood, Awrorow and Timofejewskij⁹ found that the lymphocytes, in multiplying and undergoing differentiation, were converted into various forms—macrophages, giant cells, and even spindle-shaped and stellate connective tissue cells.

It is well known that the genetic relationship between the types of connective tissue cells is very close. Sabin¹⁰ has shown lately that clasmotocytes and monocytes, that is large mononuclear leucocytes, are identical in origin. Their differentiation into fibroblasts is, therefore, a phenomenon which could easily be expected and was already considered as certain. Policard and Desplas¹¹ consider that fibroblasts derive from lymphocytes in granulating wounds. According to Dubreuil,¹² the large mononuclear leucocytes wander into and become fixed in the connective tissue, grow larger, and transform

⁴ Renaut, J., *Traité d'histologie pratique*, Paris, 1893, i, pt. 2, 968.

⁵ Carrel, A., *J. Exp. Med.*, 1912, xvi, 165.

⁶ Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.

⁷ Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 237.

⁸ Foot, N. C., *J. Exp. Med.*, 1913, xvii, 43.

⁹ Awrorow, P. P., and Timofejewskij, A. D., *Virchows Arch. path. Anat.*, 1914, ccxvi, 184.

¹⁰ Sabin, F. R., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 314.

¹¹ Policard, A., and Desplas, B., *Compt. rend. Soc. biol.*, 1917, lxxx, 745.

¹² Dubreuil, G., *Arch. anat. micr.*, 1913-14, xv, 53.

themselves into clasmotocytes or rhagiocrins. Then their segregation apparatus decreases, and they become fixed connective tissue cells. The differentiation *in vitro* of lymphocytes into large mononuclears observed by Maximoff,⁷ and of the large mononuclears into fibroblasts recorded by us, is a confirmation of the ideas of Renaut and his school,^{4,12} who consider the lymphocyte as the origin of all connective tissue cells. The transformation of large mononuclears into fibroblasts *in vitro* has more significance than the mere final demonstration of a phenomenon which was practically known. Thus, it shows that cell differentiation may occur *in vitro* under conditions which can easily be controlled. There is, therefore, a basis for the hope that the immediate cause of the change will be discovered.

The pure cultures of large lymphocytes were found to respond more readily than fibroblasts to the presence of embryonic tissue juice or serum in the medium. Lymphocytes and polymorphonuclear leucocytes displayed a similar sensitiveness to the same factors. This means that leucocytes are submitted to the influence of the two classes of substances which we found to be instrumental in increasing and inhibiting the rate of cell multiplication. The activating substances,¹³ as is well known, are contained in embryonic tissues and also in muscle, epithelial cells, and white blood corpuscles of adult animals, in the aqueous extracts of these tissues, and probably in their secretions. Their presence in a culture medium greatly increases the activity of fibroblasts,¹⁴ epithelial cells, and also leucocytes. If leucocytes could be activated *in vivo* by tissue juices as much as *in vitro*, important consequences should follow. The humors in which tissue juices or cell secretions are set free would acquire the power of increasing the activity of leucocytes, lymphocytes, and fibroblasts. Aqueous extract of inflamed connective tissue, and peritoneal exudates, containing white blood corpuscles, have been found to possess such a power.¹⁵ The death of groups of cells within a tissue would also set free their juices and stimulate the activity of leucocytes and lymphocytes. Should white cells secrete more freely or undergo leucolysis, the substances which were set free would stimulate the

¹² Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.

¹⁴ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

¹⁵ Carrel, A., unpublished experiments.

activity and multiplication of the macrophages and other cells. By this mechanism, the destruction of a number of leucocytes within the organism should automatically be followed by an increase in activity and by the multiplication of the remaining cells.

It is known that blood serum possesses the power of inhibiting the multiplication of homologous fibroblasts,¹⁶ and that leucocytes respond still more readily than fibroblasts to the retarding influence of serum *in vitro*. Very likely, serum possesses a similar action *in vivo* because the number of leucocytes would increase indefinitely if serum were not endowed with this property. As a consequence of the sensitiveness of leucocytes to serum, these cells must be more active in fluids containing serum proteins under a lower concentration. The relative activity and migration of leucocytes in interstitial lymph, inflammatory exudates, and blood may depend in some measure on this property. It is also probable that the increase of the growth-inhibiting action of serum in the course of life determines a decrease in the activity of the white cells and modifies their secretions. Possibly this is one of the mechanisms by which the profound changes brought about by age in blood serum may be related to certain diseases of the period of senescence, such as cancer. On the other hand, it must be remembered that within the organism the leucocytes are placed in a very complex medium, and that their response to the action of serum and tissue juices may be modified and even prevented by other factors.

V.

CONCLUSIONS.

1. Pure strains of mononuclear leucocytes were isolated from the blood of adult chickens and kept in active condition for nearly 3 months.
2. The cultures were composed of large mononuclear leucocytes which migrated and proliferated *in vitro* at a slower rate than fibroblasts. The cells had no tendency to form a tissue, as do fibroblasts and epithelial cells. They were much less resistant than fibroblasts.
3. Differentiation of the large mononuclears into cells assuming the appearance of fibroblasts took place under certain conditions.
4. The activity of the large mononuclears was increased by embryonic tissue juice and inhibited by homologous serum.

¹⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. 24 hour culture of leucocytes taken from the blood. In the lower left corner is seen part of the original mass of cells. The coagulum is invaded by a large number of cells. The inner zone is composed chiefly of lymphocytes and mononuclear leucocytes and the outer zone of polymorphonuclear leucocytes. The intermediate zone which appears as a darker ring is occupied by large mononuclear ameboid cells. $\times 62$.

PLATE 30.

FIG. 2. Second passage. The culture is still composed of mononuclear and polymorphonuclear leucocytes. The inner zone contains irregularly shaped, more or less elongated, ameboid cells, and the outer zone polymorphonuclear leucocytes, the number of which has decreased considerably. $\times 133$.

PLATE 31.

FIG. 3. Twelfth passage. Pure strain of large mononuclear leucocytes. The polymorphonuclear leucocytes have completely disappeared. $\times 240$.

PLATE 32.

FIGS. 4 to 6. Large mononuclear cells, with active pseudopods. $\times 1,000$.

FIGS. 7 and 8. Mitotic figures in a pure culture of large mononuclears. $\times 1,000$.

PLATE 33.

FIG. 9. Colonies of leucocytes in a Gabritschewski dish, showing their lack of coalescence. $\times 2.5$.

PLATE 34.

FIG. 10. Culture 26330, eighth passage. (a) Pure strain of mononuclear leucocytes undergoing differentiation. There are a number of mitoses scattered throughout the preparation. $\times 100$. (b) Higher magnification of the same culture. $\times 250$.

PLATE 35.

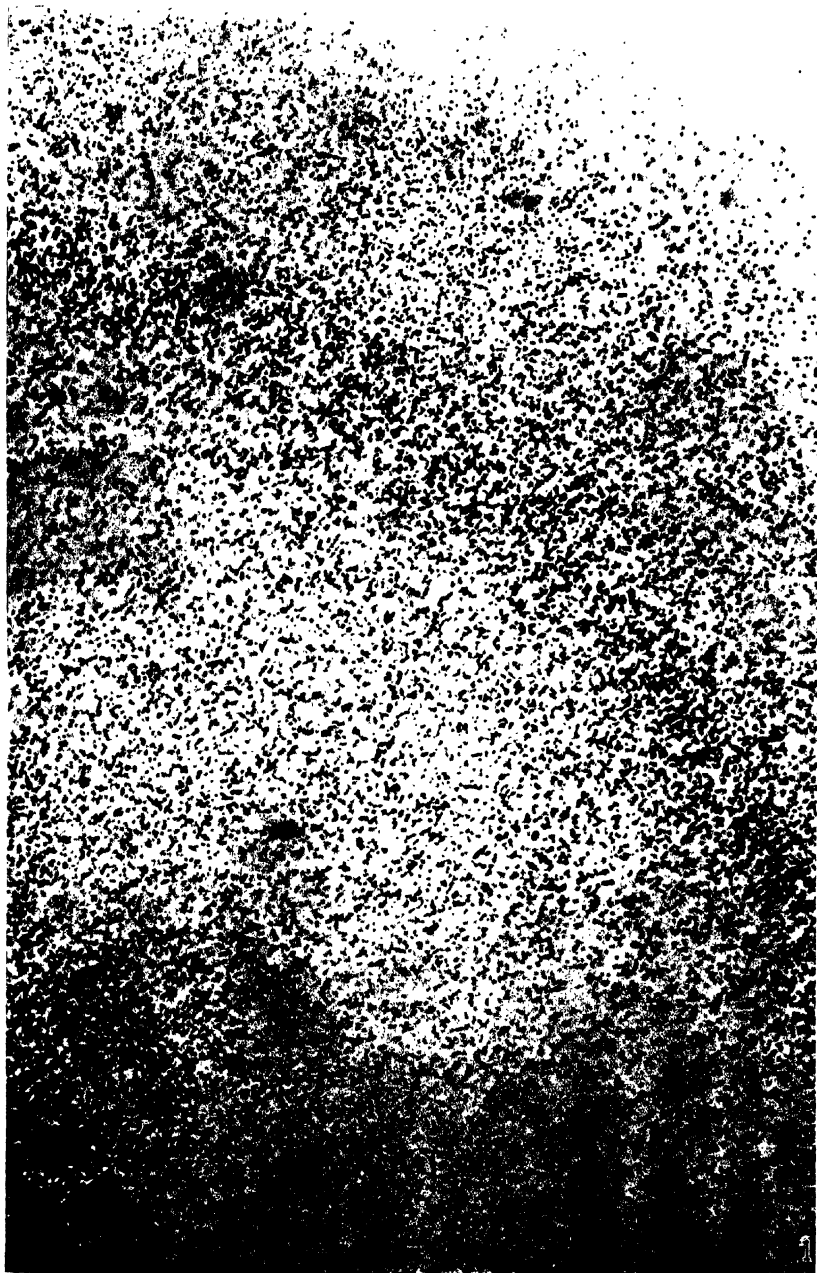
FIG. 11. Culture 26330, eighth passage. Higher magnification of two neighboring cells from the culture shown in Fig. 10. (a) Large mononuclear assuming the appearance of a fibroblast. $\times 1,000$. (b) Large mononuclear, actively ameboid. $\times 1,000$.

PLATE 36.

FIG. 12. (a to d) Large mononuclear leucocytes transformed into spindle-shaped and stellate forms. $\times 1,000$.

PLATE 37.

FIG. 13. Semidiagrammatic drawing of colonies of (a) fibroblasts and (b) leucocytes. The colonies of fibroblasts have united at their edges.



(Carrel and Ebeling: Large mononuclear leucocytes.)



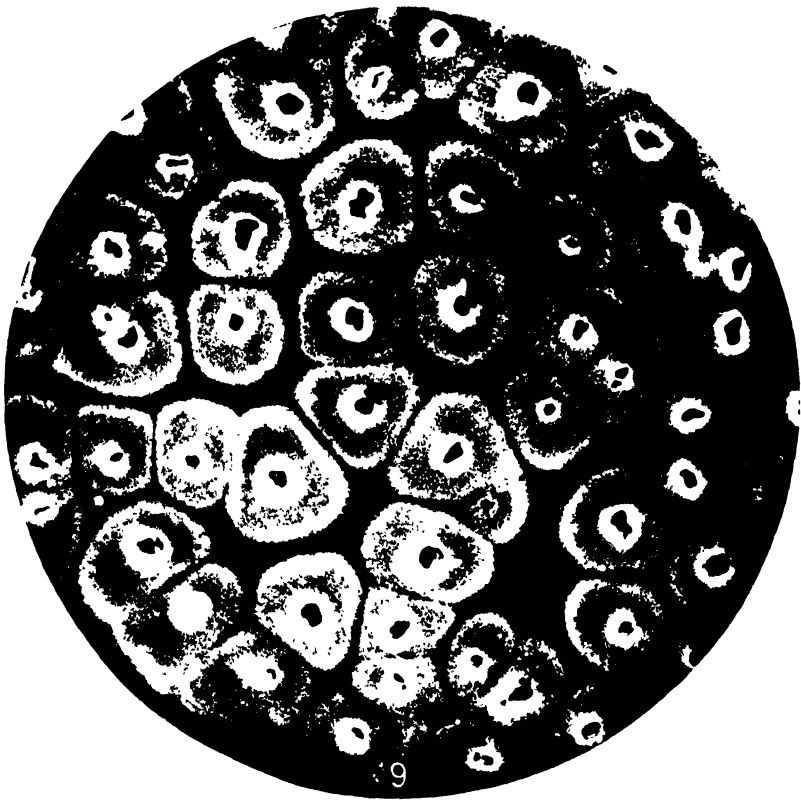
(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



(Carrel and Ebeling: Large mononuclear leucocytes.)



11a



11b

(Carrel and Ebeling: Large mononuclear leucocytes.)



12a

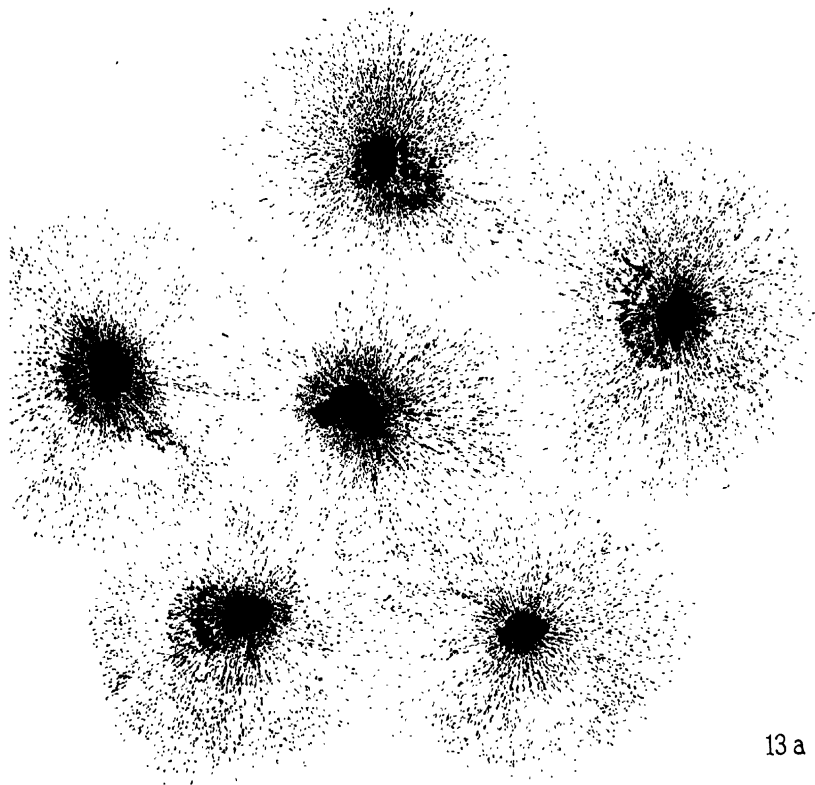
12b



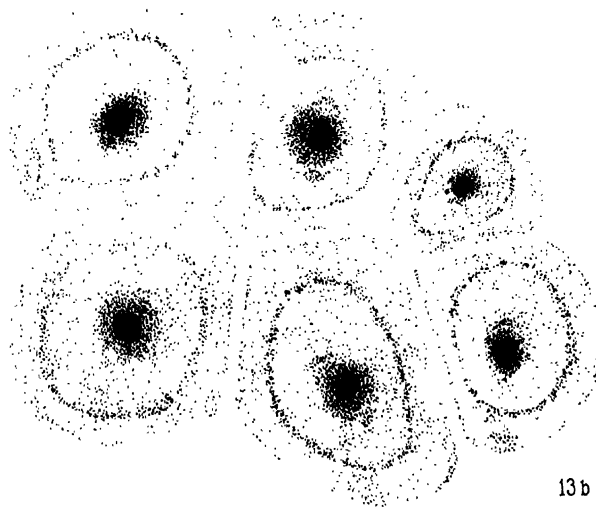
12c

12d

(Carrel and Ebeling: Large mononuclear leucocytes.)



13 a



13 b

(Carrel and Ebeling: Large mononuclear leucocytes.)

A PURE STRAIN OF CARTILAGE CELLS IN VITRO.

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PLATES 38 TO 40.

(Received for publication, April 4, 1922.)

I.

INTRODUCTION.

No pure strain of cartilage has so far been isolated. However the importance of working with pure cultures of the various types of tissue cells has been shown.¹ At present, only one method of obtaining a pure strain of a given type is known; namely, the isolation of tissues for cultivation from portions of the organism where they may be obtained free from any other tissue elements. In the eye of the chick embryo, we found an anatomical *locus electus* for obtaining pure cartilage. The sclera of the posterior hemisphere of the bulbus oculi in most birds and fish has a pars cartilago scleræ, and sometimes even a pars ossea. In sectioning the eye, it was noticed that free dissection of the cartilage could be made.

II.

EXPERIMENTAL.

1. *Isolation of the Cartilage.*—When the excised eye-bulb of the chick embryo is placed on its cornea, one may easily push aside the dark pigmented membrana nictitans with a small knife and an iris forceps; just below is seen a thin, white, semitransparent membrane. This is the cartilage. With the small forceps it may be lifted out. Immediately upon its removal, it retracts as does a drop of mucus, but when placed in a little Ringer solution, it spreads out as a thin membrane. In other respects it is not at all like mucus, but is very fragile and disintegrates easily.

¹ Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367.

2. *Technique and Description of the Cultures.*—The culture medium is the same as that generally used for connective tissue cells and epithelium, consisting of equal volumes of adult chicken plasma and juice from chick embryos. The fragments of cartilage are placed on the surface of the coagulated plasma medium. Usually the growth does not begin in a promising way, but after three to five passages it becomes very active. If the piece of cartilage is embedded in the medium, as is done in cultivating fibroblasts no growth seems to take place. The fragment of cartilage remains apparently unchanged in shape in the same medium for many days, but by measuring the area of cartilage the day the culture is made and again after 3 or 4 days, or even longer, it is found to have increased in size. It is not certain whether this increase is due to a loosening of the tissue or to a veritable increase in the number of cells. When an attempt was made to transfer such a piece of cartilage into a new medium, after it had been embedded in the coagulum for 2 or 3 days, it proved impossible. The cartilage was so fragile that the slightest touch with the knife shattered it into innumerable pieces. If one is fortunate enough to secure a small piece on the knife and put it in Ringer solution, it spreads out very quickly, closely resembling a thin membrane of paraffin in water and being just as fragile. When it is placed in the new medium and examined under the microscope, thousands of aggregates and single cells are seen spread all over the medium. This picture is strikingly like that of a culture of yeast, especially because the cells are so small and apparently spherical. Therefore, no growth seems to take place from these colonies of cells after they are embedded in the coagulum.

An attempt was then made to place one of the small pieces of cartilage on the free surface of the clotted medium. As soon as this was done, the cells began migrating and progressively became more active and formed huge cultures. Cultivated in this way, the growth was very extensive and the cultures could be multiplied. These new cells grew in some ways like epithelium, forming thin, delicate membranes, while the individual cells were in close contact. The cultures were no longer fragile but coherent and easy to extirpate and transfer to a fresh medium.

When first examined, it seemed as if a new kind of tissue had formed which, in a measure, was true. The amorphous hyaline substance

had disappeared, and the cartilage cells grew and proliferated very actively side by side, without forming any of the hyaline substance. In order to study the details of this transformation, fresh cartilage was cultivated, embedded in the clot as well as on the free surface, and the cultures were fixed and stained in different stages. It was observed that the amorphous substance was loosened and fell to pieces, and the cartilage cells became free. The space around the cells could be seen to increase in size, and finally the hyaline substance liquefied completely (Fig. 1). The cartilage cells themselves were rather small, and the cytoplasm as well as the nucleus stained deeply. When the cartilage was embedded in the clot, the cells seemed unable to grow or to develop, unless they were in very close contact. Those cells which had been cultivated for some time in the clot were all very small. Nucleus and cytoplasm, the latter often very sparsely represented, were deeply stained (Figs. 2 and 9). The cells were about the size of small lymphocytes and the resemblance was rather striking.

From the surface-cultivated cartilage, the same process of breaking down the hyaline substance took place and a different type of cells migrated. All the transition forms could be studied here, from the small, lymphocyte-like cartilage cell to the much larger cell which finally characterized the active cartilage cultures. The same phenomenon could be observed as in Fig. 1, and the free cells gained in size rather quickly; *i.e.*, their cytoplasm was spread out (Figs. 3 and 4). Some resembled the cells in Fig. 5, having a deeply stained nucleus with the chromatin located at the periphery, and cytoplasm unstained and nearly as clear as glass. After further cultivation, the cells became spindle-shaped and the cytoplasm increased more and more, the cells finally appearing as in Figs. 6 and 10. The cytoplasm often contained large vacuoles. The nucleus was spherical and usually had one large nucleolus. The nucleus itself generally stained less deeply than the cytoplasm. The cells grew in close contact, forming thin membranes.

If, in this stage, the culture is transferred to the middle of the coagulum, it grows well and does not revert to the type of small cells.

III.

DISCUSSION.

Renaut and Dubreuil² came to the conclusion that the embryonic connective tissue originated from the lymphocytes. The various functions of the connective tissue determine the peculiarities of the amorphous substances excreted. In cartilage the collagen substance (connective tissue fibrillæ) is replaced by the hyaline substance. The transformation of lymphocytes into large mononuclears and polyblasts was observed by Maximoff,³ and of large mononuclear lymphocytes into fibroblasts by Carrel and Ebeling.⁴

In our experiments, it was observed that a minute, lymphocyte-like cell type migrated out from the cartilage and became rapidly transformed into cells many times larger than the original ones. They did not produce any hyaline substance. When the size of the cells increased, great morphological and physiological changes occurred in them. The cytoplasm of the small cell type was very sparse, and the nucleus had a granular chromatin arranged in its periphery. Both nucleus and cytoplasm stained very deeply with methylene blue and azure blue. On the contrary, the cells which grew out on the surface of the coagulum were large and their cytoplasm was often filled with vacuoles. The nucleus generally had one large nucleolus and stained less deeply than the cytoplasm.

The segregation apparatus of Renaut when vitally stained with neutral red appears to be very similar to that of fibroblasts (Fig. 7). It was found that the initial growth of cartilage only took place on the free surface of the coagulum. When embedded in the coagulum, the cells were unable to adhere and no growth took place.

The strain of cartilage now under cultivation is more than 3 months old, grows very actively, and can be made to multiply. During this time it has been cultivated on the free surface of the coagulum which, at present, we find to be the best method for obtaining large cultures. Of all tissues hitherto cultivated *in vitro*, it resembles epithelium most, because of its characteristic growth in membranes (Fig. 8). The

² Renaut, J., and Dubreuil, G., *Compt. rend. Soc. biol.*, 1910, lrviii, 599.

³ Maximoff, A., *Arch. Russ. Anat., Hist. et Embryol.*, 1916, i, 105.

⁴ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

cartilage liquefies the medium much more than epithelium and connective tissue cells do.

IV.

CONCLUSIONS.

1. A strain of cartilage cells, obtained from the pars cartilago scleræ of the eye of chick embryos, has been cultivated for more than 3 months *in vitro*.

2. The initial growth of the cartilage was possible only on the free surface of the coagulum.

3. The hyaline substance disappeared during cultivation *in vitro*. The succeeding stages of a transformation from small, lymphocyte-like cells into large, spindle-shaped cells were observed. The cartilage cells were spindle-shaped and grew in close contact, forming thin membranes. In surface-grown cartilage cells, the nucleus, usually containing one large nucleolus, stained less deeply than the cytoplasm.

4. The rate of growth of cartilage was slower than that of fibroblasts and epithelium. After cultivation on the surface of the coagulum, the cartilage cells could multiply even when embedded in the coagulum. But their growth was less extensive and uniform.

EXPLANATION OF PLATES.

PLATE 38.

FIG. 1. Cartilage after 48 hours incubation in plasma medium. The amorphous substances are broken up. The cartilage cells contain relatively little cytoplasm and the nucleus and cytoplasm are stained very deeply. Stained with azure blue.

FIG. 2. Types of isolated cells of cartilage from 2 day old cultures.

FIG. 3. Types of cartilage cells after escape from the amorphous substances.

FIG. 4. Types of cartilage cells cultivated on the surface of the medium.

FIG. 5. Cell types of cartilage found in cultures of fresh cartilage after 48 hours incubation. Nuclei deeply stained, with the granular chromatin arranged in the periphery. The cytoplasm is unstained and clear as glass.

FIG. 6. Cartilage cells growing on the surface of the medium.

PLATE 39.

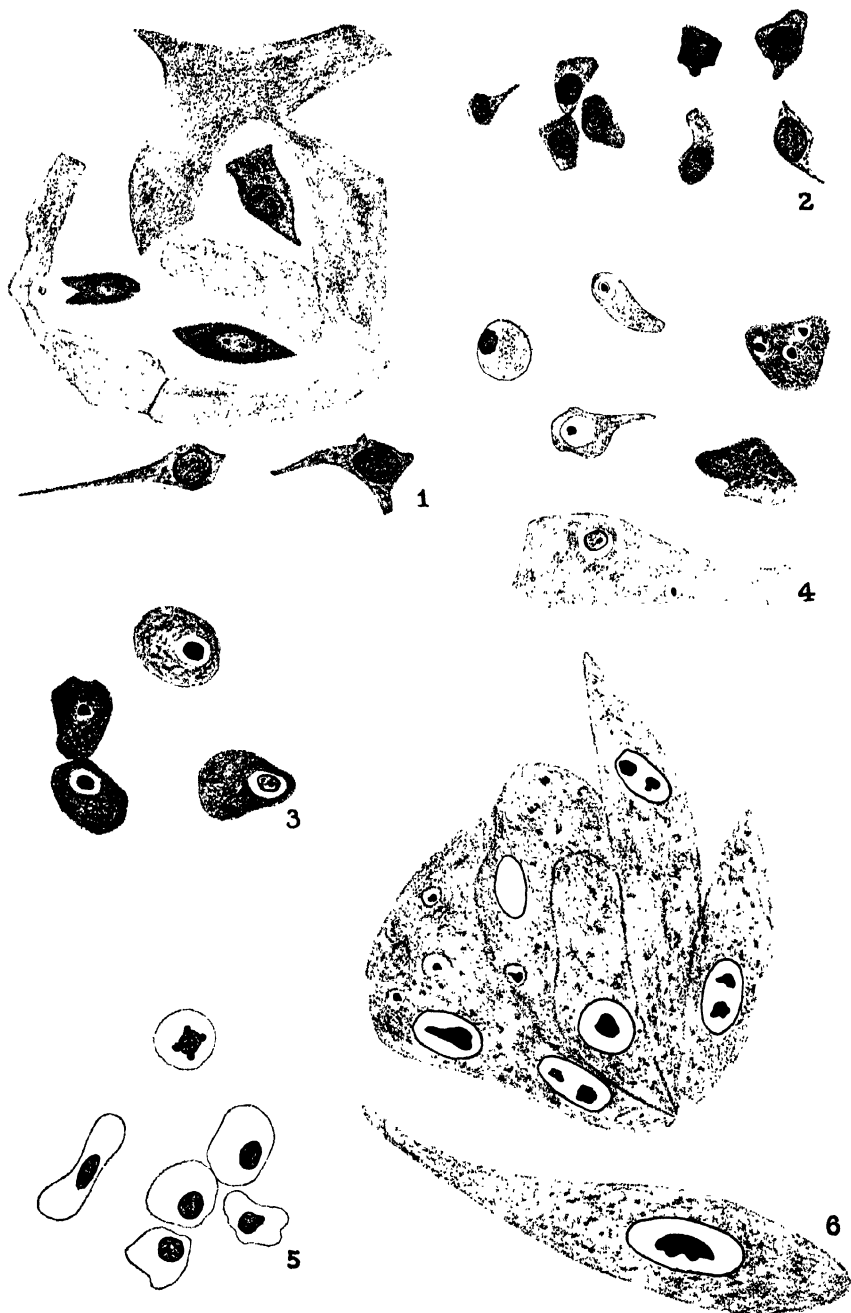
FIG. 7. The segregation apparatus of Renaut, vitally stained with neutral red. The cultures are 6 weeks old; 24 hours incubation.

FIG. 8. 2 month old culture of cartilage cells after 48 hours incubation. Stained with azure blue. $\times 60$.

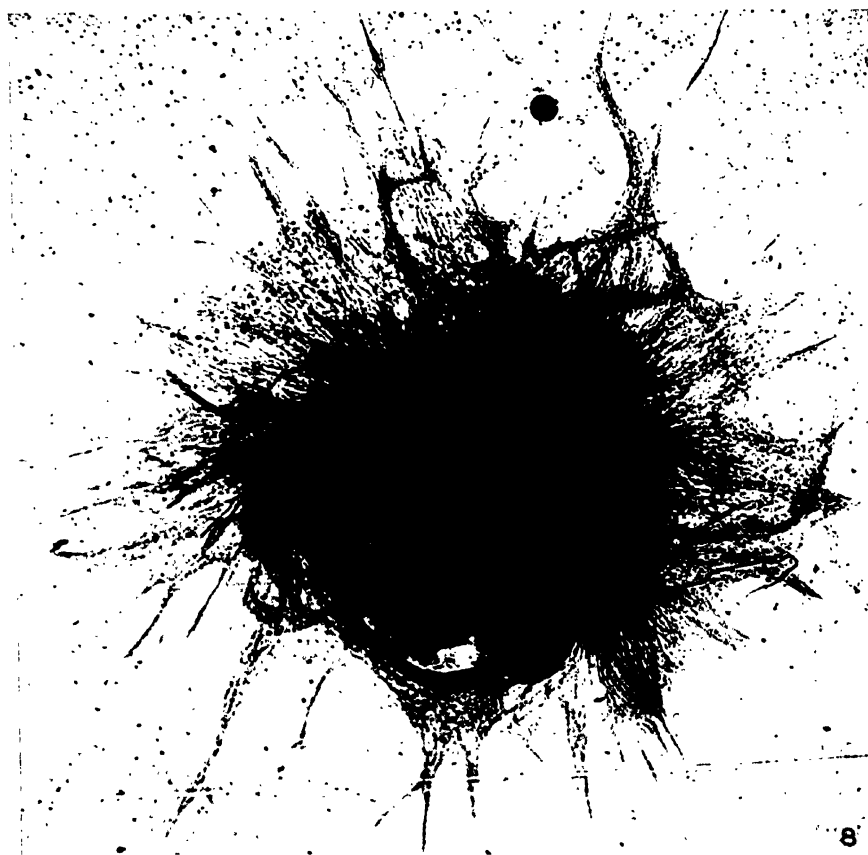
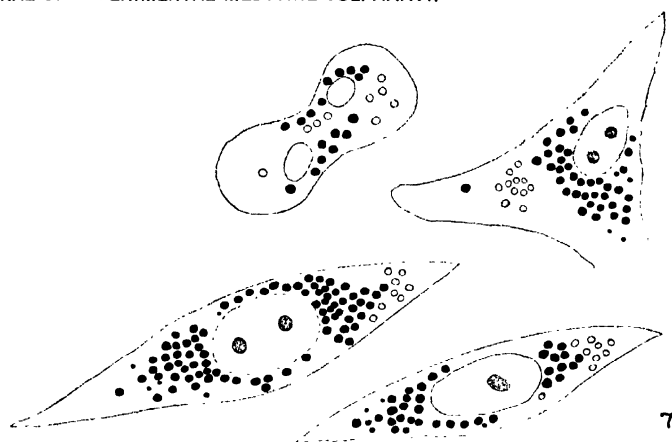
PLATE 40.

FIG. 9. Fresh cartilage cultivated on the surface of the plasma clot, showing the small types of cells just migrating on the medium. Stained with azure blue. $\times 1,000$.

FIG. 10. Experiment 1634. Mitotic figures in cartilage cells. Stained with azure blue. $\times 1,000$.



(Fischer: Pure strain of cartilage cells *in vitro*.)





(Fischer: Pure strain of cartilage cells *in vitro*.)

GROWTH-PROMOTING FUNCTION OF LEUCOCYTES.

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(Received for publication, April 10, 1922.)

It is well known that the tissues of an adult animal remain capable of resuming the activity characteristic of youth. Even in extreme old age, wounds always heal, although cicatrization may require the formation of a large amount of new tissues. We are as yet ignorant of the mechanisms which cause cells at rest in a growth-inhibiting medium, such as the humors of an old animal, to proliferate again. The activity of a cell at a given instant is function of its activity at the preceding instant and of the concentration of certain substances in its medium.¹ Therefore, it is probable that tissues which have been in a resting condition for several years cannot grow again unless they receive the food material required by the cells for their multiplication. We know that certain substances contained in embryonic juice are endowed with the remarkable property of greatly activating the rate of cell proliferation *in vitro*.^{1,2} Possibly such growth-promoting substances must be supplied to adult tissues when they cicatrize or regenerate. One of the sources of these substances may be the leucocytes which, remaining in the embryonic stage of development during the entire life of the organism, probably contain the growth-activating substances characteristic of embryonic tissues. The purpose of the experiments described in this article was to study the value of this hypothesis by ascertaining whether leucocytes contain and secrete growth-promoting substances, and whether tissues and exudates in which they accumulate acquire the power of activating cell proliferation.

¹ Carrel, A., *J. Exp. Med.*, 1913, xviii, 287.

² Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317.

I.

Growth-Promoting Action of Leucocytic Extracts and Secretions.

Leucocytes were obtained from the blood of chickens 1 or 2 years old. After centrifugation, the leucocytes were removed from the surface of the red blood corpuscles, washed several times in Ringer solution, and placed in a small amount of distilled water. After 1 or more days in the ice box, the cell suspension was shaken with once

TABLE I.

Action of an Extract of Leucocytes on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Leucocyte extract.	Embryo juice.	Ringer solution.
1	247	Embryonic heart.	247	4.0		2.0
2	247	" "	247	4.0		2.0
3	247	" "	247	5.5	6.0	
4	247	" "	247	5.0	6.0	
5	275	" "	276	5.0		2.5
6	275	" "	276	6.0		2.0
7	275	" "	288	3.5		2.0
8	275	" "	288	3.0		2.5
9	470	" "	490	4.0		2.5
10	470	" "	477	3.0		1.7
11	18488	" "	571	2.5	3.0	
12	18488	1685th passage fibroblasts.	571	4.0	3.0	
13	18488	1685th " "	575	3.0	3.0	
14	18488	1687th " "	587	5.0	6.0	
15	18488	First subculture from Experiment 14.	587	5.0	5.0	

or twice its volume of Ringer solution and centrifuged. The extracts were, therefore, more diluted than the embryonic juice generally used in tissue cultures. Their activity was tested against fragments of chick embryo heart and pure cultures of chicken fibroblasts. The medium was composed of one or two volumes of plasma and one volume of leucocytic extract. In the controls, the leucocytic extract was replaced by Ringer solution or by embryonic juice. The rate of growth was ascertained by measurements of the width of the ring of new tissue which had grown in 48 hours. Fourteen experiments were performed (Table I). Although the extracts were diluted, the

width of the growth was generally 100 per cent greater in the experiments than in the controls containing Ringer solution, and about the same as in embryonic tissue extract. It is evident that dead leucocytes set free substances which promote cell multiplication as do embryonic juices.

An investigation was then made as to whether living leucocytes may secrete growth-promoting substances such as are extracted from them after death. Long ago, growth-activating substances were found to be secreted by embryonic tissues *in vitro*.^{1,2} Pulp of embryonic tissue was cultivated in plasma and Ringer solution. The experiments were incubated, while the controls were kept in cold storage. After 1 or 2 days, the serum was extracted from the coagulum and its activity tested against fibroblasts. The activating power of the fluid extracted from the cultures preserved in cold storage was lower than that of the cultures kept in the incubator. It appeared that the tissues growing actively for a short time had modified their medium by secreting in it growth-activating substances. Similar experiments were made with leucocytes. A medium composed of practically equal parts of chicken plasma and hypotonic Tyrode solution was spread on the surface of the cover of Gabritschewski dishes. About thirty fragments of a film of chicken leucocytes were placed in the medium. The control contained no leucocytes. Both dishes were incubated for 48 hours, and large colonies of leucocytes spread into the clot. Then the serum was extracted from the coagulum and tested against a pure strain of fibroblasts. The fluid from the medium which had contained leucocytes was markedly more active. It was evident that, under the conditions of the experiments, leucocytes secreted a substance which activated the rate of growth of fibroblasts.

II.

Growth-Promoting Action of Inflamed Connective Tissue Extracts and Peritoneal Exudates.

If leucocytes be capable of setting free growth-activating substances *in vivo* as well as *in vitro*, tissues and exudates where they accumulate must acquire the power of accelerating cell multiplication. Therefore, an attempt was made to ascertain whether inflamed connective tissue contains growth-promoting substances. It is known that extracts of connective tissue stimulate cell proliferation only slightly.¹

However, control experiments were repeated. Subcutaneous connective tissue taken from a chicken was extracted with Ringer solution. The growth in the cultures containing the extract was about 25 per cent larger than in the controls containing only Ringer solution. In order that leucocytes could accumulate in the connective tissue, a focus of aseptic inflammation was produced by injecting a solution of dilute hydrochloric acid into fragments of sponge placed under the skin of chickens.³ The connective tissue around the foreign bodies became markedly thickened, although no abscess developed. Fragments of inflamed connective tissue were then removed and cultivated in plasma in order to ascertain whether they contained living leucocytes. They were soon surrounded by a large ring of ameboid cells and after 24 hours the cultures looked almost like those of Rous sarcoma. The inflamed connective tissue was sliced into a pulp, extracted with a small amount of Ringer solution, and the extract tested against fibroblasts and embryonic heart. The medium was composed of one or two volumes of plasma and one volume of extract. The controls contained Ringer solution or embryonic tissue extract. Although the inflamed tissue extracts were dilute, the width of the new tissue was more than doubled by their presence (Table II). There was no doubt that inflamed connective tissue contained substances capable of increasing the rate of multiplication of fibroblasts.

Similar experiments were repeated with peritoneal exudates. Injections of staphylococcus suspension, dog red blood corpuscles, turpentine, or bouillon into the peritoneum of chickens did not determine the production of an exudate. However, pus obtained from a turpentine abscess in a dog was injected into the peritoneum of two chickens, and an opaque fluid, orange-yellow in color, was aspirated from the abdominal cavity 2 days later. This fluid contained a large number of white blood corpuscles. After centrifugation, it was tested against embryonic heart tissue and fibroblasts. In twelve experiments, the width of new tissue was 100 per cent larger than that obtained in Ringer solution and about equal to that in embryonic juice (Table III). It was then certain that a peritoneal exudate containing many leucocytes had acquired the power of stimulating cell multiplication.

³All operations were performed under ether anesthesia.

TABLE II.

Action of an Extract of Inflamed Connective Tissue on the Rate of Multiplication of Fibroblasts.

Experiment No.	Extract No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Inflamed tissue extract.	Embryo juice.	Ringer solution.
1	213	Embryonic heart.	258	1.0		0.5
2	213	" "	258	1.5		0.5
3	213	" "	258	1.5		0.5
4	213	" "	263	4.2		2.7
5	213	1650th passage fibroblasts.	261	7.0	5.0	
6	213	1650th " "	261	5.5	6.0	
7	213	Embryonic heart.	269	3.0		1.0
8	213	" "	269	3.0		1.5
9	213	" "	269	4.0		1.5
10	311	" "	322	3.0		2.0
11	328	" "	332	4.0		2.0
12	328	" "	332	4.0		2.0
13	339	" "	349	1.5		0.8
14	339	" "	350	3.0		0.8

TABLE III.

Action of Peritoneal Exudate on the Rate of Multiplication of Fibroblasts.

Experiment No.	Exu-date No.	Nature of tissue cultivated.	Culture No.	Width of ring of new tissue.		
				Exu-date.	Em-bryo juice.	Ringer solution.
1	844	1719th passage fibroblasts.	846	5.0	5.0	
2	844	1719th " "	846	3.0	4.0	
3	844	Embryonic heart.	852	2.0		1.0
4	844	" "	852	2.0		1.0
5	850	" "	858	2.0		0.8
6	850	" "	858	1.5		0.5
7	850	" "	858	3.5		0.5
8	850	" "	858	4.5		0.5
9	850	" "	864	2.5	2.0	
10	850	" "	864	1.75	2.0	
11	866	1727th passage fibroblasts.	852	2.0		1.0
12	866	1727th " "	852	2.0		1.0

III.

DISCUSSION AND SUMMARY.

Two main facts were brought to light by the preceding experiments: first, the presence of growth-activating substances in the leucocytes; second, the setting free of these substances in tissues and fluids where leucocytes accumulate. The existence of growth-promoting substances within the body of the leucocytes was to be expected. Leucocytes are embryonic cells and it is well known that embryonic tissues contain substances which stimulate cell proliferation. But the experiments gave a direct experimental proof of this fact. Then, during the whole life and even in extreme old age, there is a supply of growth-promoting substances within the organism which is potentially capable of restoring the activity of the resting cells. Embryonic tissue juice, as is known, can rejuvenate cells which have ceased to multiply *in vitro* and show evidences of degeneration.¹ If the growth-activating substances of leucocytes can be transferred *in vivo* to tissue cells, they may play a similar rôle.

Therefore, it was important to find out whether the growth-activating substances were set free either by the secretions of the living leucocytes or by diffusion from the body of the cells after they were injured or dead, and whether this phenomenon occurred actually *in vivo*. Indeed, the idea that leucocytes secrete substances necessary to normal physiological processes is by no means new. Long ago, Ranvier described the lymph cells as mobile unicellular glands,⁴ and Renaut thought that their function was to bring to fixed cells the necessary food material.⁵ This rôle of the leucocytes was considered by him as absolutely essential. According to his theory, differentiated cells could not live in the absence of the lymph cells, which supply them with the substances required for their activity. The presence of such substances in the leucocytes was shown by our experiments and the growth-activating power acquired by inflamed connective tissue demonstrated that the leucocytes could actually bring these substances to the fixed cells.

⁴ Ranvier, cited by Renaut, J., *Traité d'histologie pratique*, Paris, 1889, i, pt. 1, 79.

⁵ Renaut, J., *Traité d'histologie pratique*, Paris, 1889, i, pt. 1, 79, 94.

Under certain conditions, this property of the white cells of the blood may cause the resumption of the activity of tissues which are in a resting state. In the adult organism, the tissues have ceased to grow and the blood plasma has acquired marked inhibiting properties. But growth-promoting substances are still stored in leucocytes, glands, and muscle tissue. The leucocytes could supply fibroblasts or epithelial cells with the necessary food material if they were present where cell proliferation is needed. The existence of mechanisms causing leucocytes to invade tissues in need of repair is certain. The initiation of healing seems to depend on the coming of the leucocytes to the wounded tissue. When they are missing, as happens when the wound is protected from all external irritation, cicatrization is greatly delayed. On the contrary, when staphylococci, turpentine, and other irritants are applied at the surface of the wound, granulations appear after less than 48 hours.⁶ These irritants, although different in nature, have the common characteristic of determining an inflammation of the tissues and the migration of leucocytes from the vessels to the surface of the wound. Possibly the white cells bring the substances which adult tissues require in order to cicatrize or regenerate. They would have the function of storing away the growth-promoting substances characteristic of embryonic tissues, and bringing them to the regions of the organism where they are needed.

IV.

CONCLUSIONS.

1. Leucocyte extracts, like embryonic tissue juice, possess the power of increasing the rate of multiplication of fibroblasts *in vitro*.
2. Leucocyte secrete substances *in vitro* which also promote cell multiplication.
3. Peritoneal exudate or connective tissue invaded by leucocytes acquires the power of increasing the rate of multiplication of fibroblasts.
4. Leucocytes are capable of bringing growth-activating substances to tissue cells. They may have the important function of promoting cell multiplication in the parts of the organism where they accumulate under certain conditions.

⁶ Carrel, A., *J. Exp. Med.*, 1921, xxxiv, 425.

CULTURES OF ORGANIZED TISSUES.

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PLATES 41 AND 42.

(Received for publication, May 31, 1922.)

It was shown by Thomson¹ that if a whole organ or a complete chick embryo 24 hours old was explanted to a culture medium, it increased markedly in size and no outgrowth of cells took place. If part of an organ, for instance a toe of a chick embryo, was explanted, an uncontrolled proliferation of cells began from the injured parts. When the basement membranes are injured, the cells begin growing out into the medium, whereas an uninjured organ continues to grow as a complete structure controlled by the laws of the organism. Thomson calls this "somatic growth." The increase of the organ stopped entirely after it reached a certain size. Possibly the cessation of growth was due to lack of absorption of the nutriment.

The experiments reported herein are in a way similar to those of Thomson. We used fragments of intestines from chick embryos which were about to hatch. At this period of embryonic development, the intestinal epithelium is assumed to possess its normal function.

I.

Technique.

The experiments were carried out in the following way. Chick embryos about 21 days old were taken out of the shell aseptically. The abdominal cavity was exposed and a fragment of the small intestine was extirpated and placed in Ringer solution. In some cases, the intestine was opened with a pair of fine scissors and a little strip was cut off and placed in the culture medium, which consisted of equal volumes of chick plasma and tissue juice. In other experi-

¹ Thomson, D., *Proc. Roy. Soc. Med.*, 1913-14, vii, Marcus Beck Lab. Rep., 71.

ments, the fragment of intestine was turned inside out, leaving the epithelium outside and the serosa inside the lumen. It was washed to free it from the meconial mucous secretions and transferred to the medium. After 48 hours incubation at 39°C., the culture chamber was opened and the fragment was picked out with the point of a cataract knife, or was aspirated in a pipette. Then it was washed for a minute or so, and transferred to a new medium. The small fragments of intestine brought about an extensive liquefaction of the clot, so that after 48 hours incubation, they were usually found floating in a cup-like excavation, surrounded with fluid.

II.

Description of the Cultures.

In some of the cultures, an outgrowth of connective tissue or epithelium from part of the fragment could be seen; in others, there was no uncontrolled growth whatsoever. After a few passages, the proliferation usually stopped and the epithelium could be seen growing all around the fragment, which was completely covered by epithelial cells after 48 hours. There was then no further uncontrolled growth. At this stage, the fragment had become spherical and its surface was as shiny as fresh mucous membrane. Under the microscope, it often appeared as a semitransparent body with a slightly denser central portion. In the early stage of life *in vitro*, numerous intestinal villi were visible and the individual cells of the cylindrical epithelium could easily be distinguished. Active peristaltic movements were observed around the free edge of the cuticula of epithelial cells. A broad, semicircular pseudopod was often observed to protrude from one cell, followed by a similar activity in the neighboring cells, whereupon the first one relaxed. It was also possible to perceive migration of the ameboid cells between the epithelial cells.

In many experiments, the active contraction of the intestinal muscles could be seen *in vitro*, even after a month's cultivation. These contractions appeared as slow as the peristaltic movement *in vivo* and were brought about by a slight cooling of the preparation.

The size of the spherical bodies, or "organisms," varied according to their age. In general, they tended to grow smaller as time went on.

When the tissue was transferred to a fresh medium, the body contracted markedly, due to cooling and irritation during the manipulation, but after incubation for an hour or two in the new medium, it relaxed and consequently increased in size. Shortly thereafter, liquefaction took place around the epithelium, which secreted a mucous fluid. In a few cases, the actual secretion could be seen to exude from the epithelial cells. At the same time, several ameboid cells could be seen wandering through the stomata of the epithelial coat. They usually deteriorated rapidly in the mucus which surrounded the fragment. In later states, the intestinal body became pellucid, and the coat of regular cubic epithelium could be studied thoroughly. Often it was possible to observe small appendicular cystic formations which appeared on the surface of the body within a few hours (Fig. 1).

III.

Histological Examination.

After a month's cultivation, the tissues were fixed in 2 per cent formol-Ringer solution, and sectioned in series. They were found in an excellent state of preservation. The cylindrical epithelium had grown all around the fragment (Fig. 2). The villi had disappeared and the surface of the body was very even and smooth. The epithelium in the deep Lieberkühn glands was well preserved in many cases, but did not seem to have any communication with the outside. The connective tissue cells had formed a stroma with numerous fibrillæ. There were no evidences of uncontrolled growth, as the epithelial cells did not go beyond their natural border-line, and there was no intermixing of the different cells.

It was supposed that bodies such as this would be able to live in a fluid medium. Therefore, fragments of intestines were cultivated in the usual way in chick plasma and tissue juice until they were entirely coated with epithelium. Then they were placed on the bottom of hollow slides and 3 or 4 drops of tissue juice were added. At the end of about a month, they were fixed, sectioned, and stained. There was a marked difference between those cultivated in the fluid medium and those in the solid medium. In the fluid medium, the villi were perfectly preserved, and very little stroma was left. In the

center of the "organism" only a faint shadow of the stroma remained. Just under the lining epithelium which appeared to be normal, were a large number of epithelial cells in a more or less ameboid state. Under this layer, an empty space could be seen which had probably been filled with liquid, and finally toward the center of the body, a very thin, loose stroma with single connective tissue cells.

The cylindrical epithelium was very well preserved. Its free surface formed a continuous and solid cuticula. This contact between the epithelial cells was only perfect in their peripheral part. Between the external and the basal parts of the cells, free spaces could be seen, as if they had been formed under the pressure of a liquid within the body. The epithelial border of one which had been kept in a fluid medium for a long time is shown in Fig. 3.

IV.

DISCUSSION AND SUMMARY.

An artificial organism, if one may so term it, composed of a complex of tissues, was cultivated for a long period of time. Small fragments of intestine from chick embryos 20 to 21 days old were placed in a suitable medium. The epithelium proliferated and completely covered the fragment of intestine after 4 to 6 days. A small body was thus formed, round or oblong in shape, surrounded by cylindrical epithelium and containing epithelial, connective, and muscle tissues, endothelium, and ameboid cells. After a month's cultivation *in vitro*, no necrosis had occurred. Therefore, it may be assumed that, through the intestinal epithelium, the medium supplied the intestinal tissue with sufficient nourishment. No uncontrolled proliferation took place after the epithelium had surrounded the entire fragment.

The cultivation of complex tissues will facilitate the study of the interactions of the different cells under various conditions. In some experiments, pure cultures of epithelial cells were grafted into such an "organism" without difficulty. The growth of malignant cells could be studied in the same way. When the "organism" was placed in a fluid medium, the epithelium remained normal but the stroma disappeared. It seems that plasma played an important rôle in the maintenance of the tissues in their normal condition.

V.

CONCLUSIONS.

1. Fragments of small intestine from a 21 day old chick embryo, cultivated in plasma and tissue juice, became completely surrounded with cylindrical epithelium.

2. After a month's cultivation in plasma and tissue juice, the tissues composing the mass were normal. It would seem that the necessary food material was absorbed by the epithelium from the culture medium.

3. When these masses were cultivated in embryo juice without plasma, the intestinal villi remained normal, while the stroma, connective tissue cells, and their fibrillæ progressively disappeared, leaving an epithelial cyst.

EXPLANATION OF PLATES.

PLATE 41.

FIG. 1. Experiment 2107-24. Section of an intestinal "organism" cultivated in embryonic juice without plasma. The epithelium is normal. The stroma is very loose and the cells are sparsely represented. A cystic formation is seen at A. $\times 150$.

FIG. 2. Experiment 1085-6. Section of an intestinal "organism" cultivated in plasma and embryonic juice for a month. $\times 200$.

PLATE 42.

FIG. 3. Experiment 2107-22. Section of an intestinal "organism" cultivated in embryonic tissue juice for a month. The loose stroma and the cubic epithelium with the dilatated spaces between the individual cells may be seen. $\times 272$.



FIG. 1.



FIG. 2.

(Fischer: Cultures of organized tissues.)



FIG. 3.

(Fischer: Cultures of organized tissues)

ACTION OF SHAKEN SERUM ON HOMOLOGOUS FIBROBLASTS.

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(Received for publication, June 1, 1922.)

The activity of homologous fibroblasts decreases after the serum composing their medium has been heated at 56° and 70°C., while under the same cultural conditions, the rate of migration of heterologous fibroblasts increases.¹ The better growth of heterologous tissue in heated serum is probably due to the destruction of alexin. But the cause of the decreased activity of homologous tissue is still unknown. It may be attributed to the disappearance of alexin or of some other factor which should have, in common with alexin, the property of being thermolabile. Therefore, an attempt was made to ascertain whether factors other than heat might bring about simultaneous changes in the inhibiting action of serum on homologous fibroblasts, and in its alexinic activity. The purpose of the experiments described in this article was to study the effect of serum inactivated by shaking, on the growth of fibroblasts.

I.

Action of Shaken Serum on Foreign Erythrocytes and on Homologous Fibroblasts.

Serum was obtained from plasma of chickens about 1 year old, sealed in Pyrex tubes, and shaken at low speed for periods varying from 1 to 8 hours. It became more or less opalescent and, after centrifugation, a slight precipitate settled at the bottom of the tube. The volume of the precipitate varied according to individual differences in the animals. It was never very large, and the variations of the refractive index of the serum before and after shaking were

¹ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

always slight. There was practically no difference in the H ion concentration of the fluid. The lytic action of serum on foreign cells was ascertained with sheep corpuscles. Fibroblasts obtained from

TABLE I.
Action of Shaken Chicken Serum on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		<i>hrs.</i>				<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1406	1	1445	2.00	1.50	20	10	5
		1	1445	2.00	1.00	50	50	18
		1	1445	4.00	2.00			
		1	1445	3.00	1.50	70	90	35
2	1429	7	24967	3.48	2.97			
		7	24967	2.40	1.67			
		7	24967	3.59	2.50			
		7	25071	2.80	2.70			
		7	25071	3.54	2.89			
		7	25071	3.82	1.69			
3	1737	4	1741	4.76	1.39			
		4	1741	3.47	0.83			
		4	28374	3.36	2.62			
		4	28374	3.28	2.11			
		4	28374	3.21	2.72			
		4	28374	2.50	1.76			
4	1737	8	1750	1.60	0.01	10	45	0
		8	1750	2.26	0.01	20	100	0
5	1746	4	1751	3.19	1.82	20	80	70
		4	1751	3.90	1.01			
		4	1751	5.50	5.30	30	100	100
6	1758	4	1761	3.00	2.00			
		4	1761	2.00	1.50			
7	1758	7	1798	5.41	4.50	10	10	5
		7	1798	3.59	1.00	20	40	15
		7	1798	0	0	40	100	35

pure cultures of a 10 year old strain were used for measuring the effect of chicken serum on homologous cells. The medium consisted of one volume of normal plasma, two volumes of shaken serum, and one volume of shaken serum containing 1:20 embryo juice. In the controls, the shaken serum was replaced by normal serum. The rate of growth was measured according to a technique previously described.²

Five sera, shaken from 1 to 8 hours, were examined (Table I). They were partly inactivated as shown by the hemolytic tests. In one case, inactivation was complete. The inhibiting action of shaken serum on fibroblasts was always found to be at least 30 per cent greater than that of unshaken serum. In the experiment in which serum was completely inactivated, the differences in the action of both shaken and unshaken sera were still more marked (Table I, Experiment 4). It was evident that partial or complete inactivation by shaking had a marked restraining effect on the activity of homologous fibroblasts.

II.

Action of Shaken Serum on Foreign Erythrocytes and on Foreign Fibroblasts.

Fresh dog serum, shaken in the same manner as chicken serum, became slightly opaque and partly inactivated (Table II). Its in-

TABLE II.

Action of Shaken Dog Serum on Chicken Fibroblasts and Sheep Erythrocytes.

Experiment No.	Serum No.	Time shaken.	Culture No.	Rate of growth of fibroblasts.		Hemolysis of sheep corpuscles.		
				Normal serum.	Shaken serum.	Concentration of serum.	Amount of hemolysis.	
							Normal serum.	Shaken serum.
		<i>hrs.</i>				<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	1396	3	1402	3.5	4.5	10	100	25
		3	1402	2.5	4.0	20	85	7
2	1300	4	1423	2.0	8.0			
		4	1423	3.0	7.0			
3	1428	7	1434	2.0	3.5			
		7	1434	2.0	3.0			

² Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

hibiting action was tested upon chicken fibroblasts taken from stock cultures of a 10 year old strain, and found very much decreased. The average width of the ring of new tissue was about twice as large in shaken as in unshaken serum. It appeared that dog serum partly inactivated by shaking became a much better medium for chicken fibroblasts.

III.

DISCUSSION AND CONCLUSIONS.

The preceding experiments showed that partial or complete inactivation of serum by shaking brought about a marked decrease in the activity of homologous fibroblasts. Generally, serum was not completely inactivated by shaking. In only one instance was the hemolytic effect of chicken serum on sheep corpuscles entirely lost after the serum had been shaken for 8 hours. In all other cases, the normal hemolytic power of chicken and dog sera for sheep erythrocytes was merely decreased. The effect of shaking varied according to certain conditions of the serum. It may be compared to the influence of heat, which differs widely according to individual sera, even when they are obtained from animals which are apparently in identical condition. Shaken serum always inhibited the activity of homologous fibroblasts more than normal serum. When chicken serum was completely inactivated by shaking, its restraining action on chicken fibroblasts became also more marked. On the contrary, dog serum partly inactivated by shaking was much less toxic for chicken fibroblasts than normal serum. Thus, shaking brought about a change in the condition of serum, against which homologous and heterologous fibroblasts reacted in an opposite manner. At the same time, the normal lytic action of serum on foreign erythrocytes decreased.

This last phenomenon is caused, as is well known, by the partial or complete destruction of alexin. The decrease of the restraining effect of shaken serum on foreign fibroblasts may be attributed to the same cause. The increase of the inhibiting action of shaken serum on homologous fibroblasts is due possibly to the disappearance of a substance favoring the activity of homologous cells. A similar hypothesis was advanced for explaining the increase of the growth-

inhibiting action of serum under the influence of senescence³ and of heat.¹ The restraining power of adult serum on cell multiplication may be due to the antagonistic action of growth-promoting and growth-inhibiting substances, the growth-promoting substance being as unstable as alexin and certain tissue juices which have the property of increasing the rate of cell proliferation. Alexin and growth-activating substances contained in embryonic and gland tissue juices have in common the property of being destroyed by heat and by shaking. Leucocytes added to serum under certain conditions increase its hemolytic power on foreign erythrocytes and decrease its inhibiting action on homologous fibroblasts. Variations in the alexinic activity of serum under other influences are followed also by a change in its action on homologous cells. Long ago, Gengou⁴ found that serum from plasma is less bactericidal than serum from blood. We observed that the lytic effect on sheep erythrocytes of dog and chicken serum from blood was sometimes more marked than that of serum from plasma, but there was often no difference in the action of both sera. At the same time, the growth of homologous fibroblasts was always more extensive in the serum from blood. The opposite effect was observed when heterologous fibroblasts were used. The growth-inhibiting power of serum from blood was still less marked on leucocytes than on fibroblasts. But embryonic juice, which greatly enhances the rate of multiplication of homologous cells, did not increase the lytic action of serum on foreign erythrocytes.

It may be concluded that, under the conditions of the experiments:

1. Chicken serum partly or completely inactivated by shaking becomes more inhibiting for chicken fibroblasts.
2. Dog serum partly inactivated by shaking becomes less inhibiting for chicken fibroblasts.

³ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

⁴ Gengou, O., *Ann. Inst. Pasteur*, 1901, xv, 232.

ACTION OF ANTIGEN ON FIBROBLASTS IN VITRO. II.

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(Received for publication, June 10, 1922.)

It is known that a strain of fibroblasts responds to the presence of an antigen in its culture medium by becoming immunized against the action of the antigen.¹ The purpose of this paper is to report further experiments in which the influence of the antigen concentration on the process of immunization of fibroblasts *in vitro* was studied.

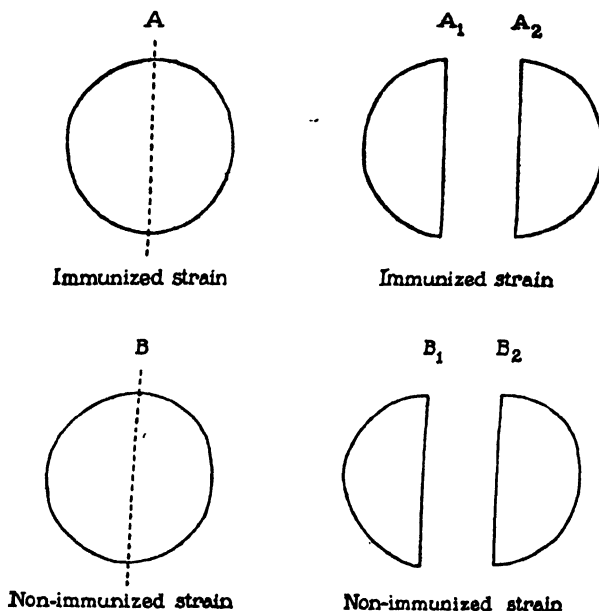
I.

Technique.

The technique was the same as described previously.¹ Dog sera and human ascitic fluid were used as antigen. The antigen was added to chick embryo juice in such a proportion that one volume of the mixture and one volume of chicken plasma would give the desired concentration. Each passage represented 48 hours, after which time the cultures were measured. The experiments were made in the following way. A fragment of tissue taken from a 10 year old strain of fibroblasts was divided into two equal parts. One was cultivated in a medium with antigen (Text-fig. 1, *A*); the other was cultivated in a medium without antigen (Text-fig. 1, *B*) and served as a control. After 48 hours incubation, the experimental and control cultures were divided into two parts. One-half of the experimental culture was transferred to a fresh medium with antigen (Text-fig. 1, *A*₁), and one-half of the control culture (Text-fig. 1, *B*₁) to a fresh medium without antigen. Both experimental and control tissues underwent many passages in media with and without antigen. The remaining halves (Text-fig. 1, *A*₂ and *B*₂) of the experimental and control cultures were placed in a medium containing the antigen under a high concen-

¹ Fischer, A.. *J. Exp. Med.*, 1922, xxxv, 661.

tration, from 50 to 66 per cent, which had a marked inhibitory action on the growth of normal tissues. In this way, it was possible to ascertain the resistance gained by the immunized tissue at a given time during the experiment. If the tissue grown in a medium containing a small amount of antigen constantly did not become immunized, its rate of growth was as slow as that of the control tissue



TEXT-FIG. 1. *A* is a culture with a small amount of antigen. *A*₁ and *A*₂ show the same culture divided in half. *A*₁ is carried on in a medium containing a small amount of antigen; *A*₂ (subculture) is transferred to a medium containing the antigen in a high concentration. *B* is the control culture. *B*₁ is carried on as *B*, the control culture, and *B*₂ is transferred to a medium containing the antigen in a high concentration. The quotient of *A*₂ divided by *B*₂ expresses the degree of immunization of *A*₁.

when both were exposed to a high concentration of antigen. Therefore, the quotient of the *rate of growth of the experimental subculture* (immunized strain in the high concentration of antigen) divided by the *rate of growth of the control subculture* (non-immunized strain in the high concentration of antigen) expressed the degree of immunization. For instance, if both immunized and non-immunized strains grew at

the same rate in a high concentration of antigen, the quotient was 1, and no immunization had taken place. If the immunized strain grew more actively than the non-immunized strain, the quotient was higher than 1 and expressed the degree of immunization.

The quotient of the rate of growth in their respective media of the immunized strain divided by that of the non-immunized strain expressed the effect of the small amount of antigen in which the tissues were constantly cultivated. A quotient below 1 indicated that antigen, even in a low concentration, inhibited the growth of fibroblasts. When the quotient was 1, antigen, in the concentration used, had no effect on the fibroblasts.

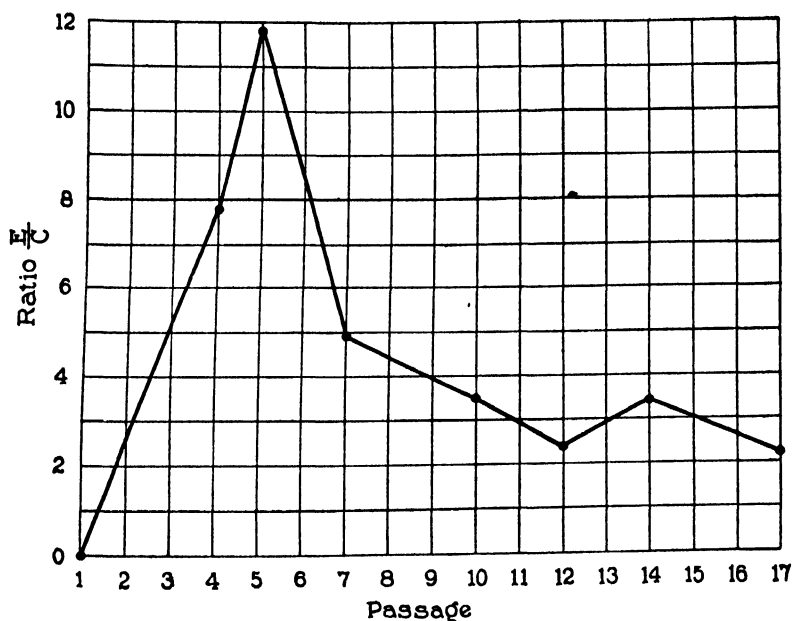
The quotient of the rates of growth of the two halves $\frac{A_1}{A_2}$ (Text-fig. 1) of the immunized strain in media containing antigen in high and low concentration expressed the effect of antigen on the immunized fibroblasts. The quotient of the rates of growth of the two halves $\frac{B_1}{B_2}$ (Text-fig. 1) of the control in media containing a high concentration and no antigen showed the effect of antigen on normal fibroblasts. These quotients were plotted in ordinates and the curves indicate that the rate of growth of the immunized strain was higher than that of the non-immunized.

II.

EXPERIMENTAL.

A. Effect of Human Ascitic Fluid as Antigen.

1. *Time and Degree of Immunization of Fibroblasts under the Influence of a Small Amount of Antigen.*—The total nitrogen of the human ascitic fluid used as an antigen amounted to 0.203 per cent. The culture medium of the immunized strain contained about 7 per cent ascitic fluid throughout the whole experiment. The results of an experiment which lasted for seventeen passages are recorded in Text-fig. 2. According to the diagram (Text-fig. 1), the ordinates in Text-fig. 2 represent $\frac{A_1}{B_1}$ (Table I), which is the rate of growth of both the immunized and non-immunized strains in high concentra-

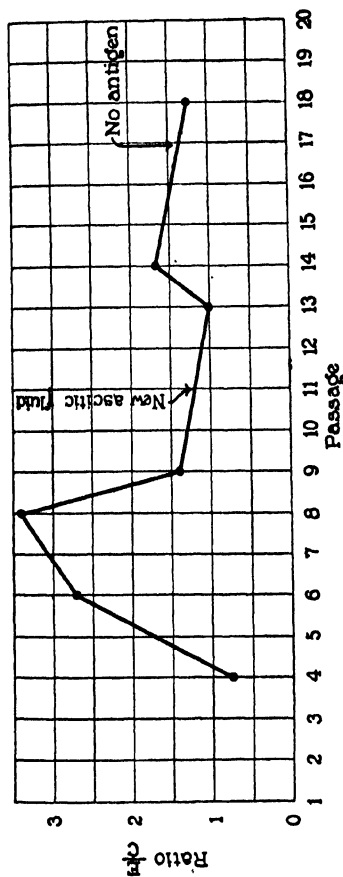


TEXT-FIG. 2. Curve showing the variations of the degree of immunization of fibroblasts through seventeen passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

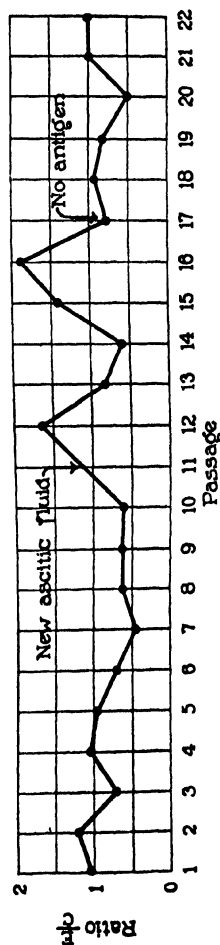
TABLE I.

Variations in the Degree of Immunization of Fibroblasts; Human Ascitic Fluid Used as Antigen in a Concentration of 7 Per Cent.

Culture No.	Passage No.	Relative increase of subcultures in 50 per cent ascitic fluid.		Ratio $\left(\frac{E}{C}\right)$ of subcultures.
		Control.	Experiment.	
847-2, 848-2	4	1.8	14.1	7.8
316-2, 317-2	5	1.1	13.0	11.8
610-2, 611-2	7	2.8	13.8	4.9
659-2, 660-2	10	4.6	16.2	3.5
686-2, 687-2	12	9.4	23.2	2.4
718-2, 719-2	14	6.0	23.0	3.4
760-2, 761-2	17	3.8	8.4	2.2



TEXT-FIG. 3. Curve showing the degree of immunization of fibroblasts through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissae the number of passages.



TEXT-FIG. 4. Curve showing the rate of growth of the immunized strain and its control through twenty-two passages. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of its control, and the abscissae the number of passages.

tions; namely, the degree of immunization. The maximum of resistance occurred at the fifth passage (10 to 12 days after the beginning of the experiment). Afterwards, the resistance declined slowly

TABLE II.

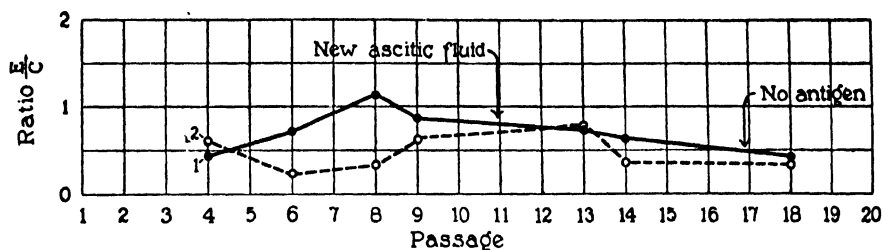
Reaction in Function of Time of Immunized and Non-Immunized Strains to a Large Amount of Antigen.

Culture No.	Passage No.	Ratio.			
		Immunized and non-immunized strains.	Non-immunized strain and its sub-culture.	Immunized strain and its sub-culture.	Subcultures of immunized and non-immunized strains.
1855-1, 1855-2	1	1.08			
1882, 1883	2	1.20			
1906, 1907	3	0.72			
1940-1, 1940-2 } 1941-1, 1941-2 }	4	1.04	0.60	0.44	0.73
1972, 1973	5	0.98			
1998-1, 1998-2 } 1999-1, 1999-2 }	6	0.70	0.28	0.70	2.70
2024, 2025	7	0.47			
2047-1, 2047-2 } 2048-1, 2048-2 }	8	0.62	0.34	1.14	3.40
2073-1, 2073-2 } 2074-1, 2074-2 }	9	0.62	0.61	0.86	1.40
2100, 2101	10	0.60			
2122, 2123	11				
2147, 2148*	12	1.63			
2170-1, 2170-2 } 2171-1, 2171-2 }	13	0.81	0.78	0.79	1.00
2199-1, 2199-2 } 2200-1, 2200-2 }	14	0.60	0.37	0.62	1.68
2217, 2218	15	1.46			
2238, 2239	16	1.90			
2266, 2267†	17	0.80			
2290-1, 2290-2 } 2291-1, 2291-2 }	18	0.94	0.32	0.40	1.25
2311, 2312	19	0.84			
2337-1, 2338	20	0.53			
2363, 2364	21	1.00			
2390, 2391	22	1.10			

* In Cultures 2147 to 2239, new ascitic fluid was used.

† In Cultures 2266 to 2391, the antigen was omitted.

and persisted still after seventeen passages (about 40 days). Another experiment of the same kind was carried through twenty-two passages. Human ascitic fluid was present continuously in a concentration of about 7 per cent in the medium of the immunized strain (Text-fig. 3 and Table II). The curve (Text-fig. 3) shows the variations of the resistance of the immunized strain. The resistance quickly reached its maximum (Text-fig. 3). Afterward, it declined, but a second maximum occurred after the thirteenth passage. The curves representing this and other experiments remind one of those obtained by Jörgensen and Madsen² for the amount of agglutinin produced by a goat under the influence of continuous injections of cholera bacilli.



TEXT-FIG. 5. Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration of antigen. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in the ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Human ascitic fluid was used as antigen in a concentration of 7 per cent. The ordinates represent the quotient of the rates of growth, and the abscissæ the number of passages.

2. Comparison of the Rates of Growth of Immunized and Non-Immunized Strains.—The immunized strain was cultivated exactly as was the control, with the exception of the addition of 7 per cent ascitic fluid (Text-fig. 4). If the antigen at this low concentration should have any growth-inhibiting effect, the quotient of the rates of growth of the immunized strain and its control would be below 1. As the average value of the quotient was 1, it appears that the antigen did

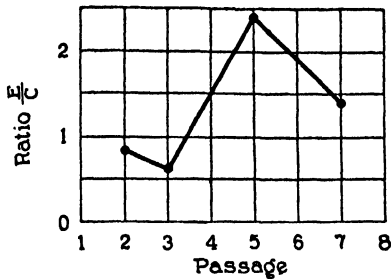
² Jörgensen, A., and Madsen, T., *Festskrift ved Indvielsen af Statens Serum Institut, Copenhagen, 1902, Paper 6, p. 12.*

not markedly affect the activity of the strain. The variations in the quotient of the rates of growth of both subcultures of the immunized strain in low and high antigen concentrations are shown in Text-fig. 5, Curve 1. The quotients of the rates of growth of both subcultures of the non-immunized strain without antigen and in a high concentration of antigen are also shown in Text-fig. 5, Curve 2. These two curves indicate that the immunized strain grew more rapidly than the non-immunized. This has been the case in all the experiments. It seems that the presence of the antigen stimulated the growth of the fibroblasts during the entire experiment.

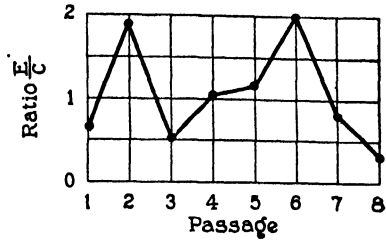
In the experiments recorded in Text-figs. 3 to 5 and Table II, new ascitic fluid was used at the eleventh passage. At the seventeenth passage, the antigen was omitted, and no change in the rate of growth occurred.

B. Effect of Dog Serum as Antigen.

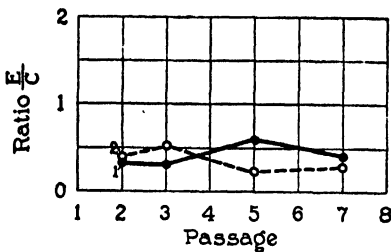
In the following experiments, dog serum was used as an antigen. When added in small quantity to the culture medium of the fibroblasts, it acted as did ascitic fluid,¹ and the fibroblasts became immunized against its action. Certain dog sera were more inhibiting for the growth of fibroblasts than others. In previous experiments,¹ the non-immunized strain of fibroblasts was often killed by a concentration of about 66 per cent, while the immunized strain remained normal. An investigation was made of the relation between the degree of immunization of a strain of fibroblasts, and the concentration of dog serum used as an antigen. Dog sera were selected which inhibited the growth of fibroblasts very markedly. The technique was similar to that previously described.¹ Fragments of a 10 year old strain of fibroblasts were immunized with antigen in different concentrations (Text-figs. 6 to 10 and Tables III to V). The antigen used was dog serum (No. 1430), in a concentration of 4 per cent. The growth index of the subcultures $\left(\frac{A_2}{B_2}\right)$, which expresses the degree of immunity, reached a maximum at the fifth passage (Text-fig. 6). The ratio between the immunized and non-immunized strains $\left(\frac{A_1}{B_1}\right)$ shows (Text-fig. 7) that, at the seventh and eighth passages, the growth index



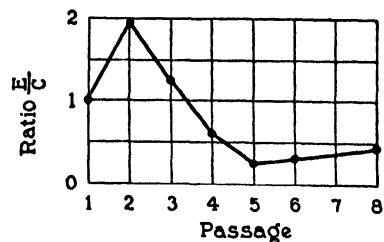
TEXT-FIG. 6.



TEXT-FIG. 7.



TEXT-FIG. 8.



TEXT-FIG. 9.

TEXT-FIG. 6. Curve showing the variations in the degree of immunization of fibroblasts through eight passages. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by that of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

TEXT-FIG. 7. Curve showing the action of antigen on the immunized strain through eight passages. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of its control, and the abscissæ the number of passages.

TEXT-FIG. 8. Curve 1 shows the variations in the quotient of the rate of growth of the immunized strain in a small amount of antigen divided by the rate of growth of the same strain in a high concentration. Curve 2 shows the variations in the quotient of the rate of growth of the non-immunized strain in the ordinary medium divided by the rate of growth of the same strain in a high concentration of antigen. Dog serum was used as antigen in a concentration of 4 per cent. The ordinates represent the quotient of the rates of growth, and the abscissæ the number of passages.

TEXT-FIG. 9. Curve showing the variations in the degree of immunization of fibroblasts through eight passages. Dog serum was used as antigen in a concentration of 8 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

TABLE III.

Immunization of Fibroblasts by Dog Serum in 4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio.			
		Immunized and non-immunized strains.	Non-immunized strain and its sub-culture.	Immunized strain and its sub-culture.	Subcultures of immunized and non-immunized strains.
1180, 1181	1	0.68			
1216-1, 1216-2}	2	1.90	0.43	0.36	0.86
1217-1, 1217-2}					
1255-1, 1255-2}	3	0.51	0.50	0.30	0.60
1256-1, 1256-2}					
1289, 1290	4	1.03			
1326-1, 1326-2}	5	1.17	0.25	0.59	2.40
1327-1, 1327-2}					
1361, 1362	6	2.00			
1397-1, 1397-2}	7	0.80	0.30	0.41	1.40
1398-1, 1398-2}					
1452, 1453	8	0.30			

TABLE IV.

Immunization of Fibroblasts by Dog Serum in 8 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1491-2, 1492-2	2	1.95
1713-2, 1714-2	3	1.25
1489-2, 1490-2	4	0.60
1538-2, 1539-2	5	0.25
1094-2, 1095-2	6	0.30
1170-2, 1171-2	8	0.45

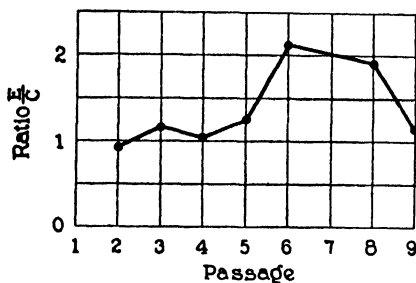
TABLE V.

Immunisation of Fibroblasts by Dog Serum in 1.4 Per Cent Concentration of Antigen.

Culture No.	Passage No.	Ratio of subcultures of immunized and non-immunized strains.
1118-2, 1119-2	2	0.92
1257-2, 1258-2	3	1.14
1602-2, 1603-2	4	1.06
1670-2, 1671-2	5	1.23
1637-2, 1638-2	6	2.13
1456-2, 1457-2	8	1.90
1908-2, 1909-2	9	1.17

being under 1, the growth of the immunized strain was very much inhibited. It indicates also that after a few passages (Text-fig. 8) the immunized strain grew more actively than the non-immunized strain.

When the antigen (Dog Serum 1430) was used in a concentration of about 8 per cent, the maximum of resistance was reached at the second passage and lasted only a short time (Text-fig. 9 and Table IV). Afterwards, the resistance fell rapidly below 1. This experiment demonstrated also that, in spite of the marked inhibiting effect of an 8 per cent concentration of antigen, the strain presented a much higher resistance against dog serum than the control immediately after the second passage. When 1.4 per cent antigen was added to



TEXT-FIG. 10. Curve showing the variations in the degree of immunization of fibroblasts through nine passages. Dog serum was used as antigen in a concentration of 1.4 per cent. The ordinates represent the quotient of the rate of growth of the immunized strain divided by the rate of growth of the non-immunized strain in a high concentration of antigen, and the abscissæ the number of passages.

the culture medium, a different result was obtained (Text-fig. 10 and Table V). This low concentration of antigen restrained the growth of the immunized strain, but was less toxic for the fibroblasts than a 5 to 8 per cent concentration. The resistance reached its maximum at the sixth or eighth passage and decreased slowly afterwards.

III.

SUMMARY AND CONCLUSIONS.

The action of human ascitic fluid and of dog serum on the immunization of fibroblasts *in vitro* was investigated. The toxic effect of ascitic fluid upon fibroblasts was slight, while that of dog serum was

marked. When a small amount of antigen (1.5 to 2 per cent dog serum, or 8 per cent ascitic fluid) was present in the medium, the resistance of the fibroblasts slowly increased, reached its maximum after about 14 days, and gradually decreased. The curve expressing the phenomena closely resembles that of Jørgensen and Madsen showing the production of antibodies in an animal which received daily injections of antigen. When the antigen was present in the medium in a high concentration (from 5 to 8 per cent dog serum), the resistance reached its maximum in 4 days, and decreased rapidly.

It may be concluded that:

1. In the immunization of fibroblasts *in vitro* against a foreign protein, there is a relation between the amount of antigen, the time of the appearance of immunization, and its duration.
2. When a small amount of antigen is used, immunization slowly reaches its maximum, and slowly decreases.
3. When a large amount of antigen is used, immunization reaches its maximum in a short time, but the protection is of equally short duration.

I wish to acknowledge my indebtedness to Dr. Alexis Carrel for his permission to use the old strain of fibroblasts. I also wish to express my gratitude for the help and kindness of Dr. Carrel and his associates during my stay at The Rockefeller Institute.

SURFACE TENSION OF SERUM.

IV. ACTION OF TEMPERATURE.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 50 TO 53.

(Received for publication, May 25, 1922.)

The action of heat on the surface tension of serum has been reported in previous papers,^{1,2} but only at a temperature of 55°C., and for a short time. The purpose of this paper is to show the effect of prolonged heat at 55°C. on the initial value of the surface tension of pure serum, and that of temperatures of 55°, 70°, and 100°C. on the time-drop of serum solutions. In order to save space, it is assumed that the reader is familiar with the technique and terms described and used in the preceding articles.

I.

Pure Serum.

In the case of pure serum, only the initial value of the surface tension was measured, because the changes in the time-drop would be almost of the order of magnitude allowed for experimental errors. Dog serum was kept in an incubator at 55°C. for 168 hours (7 days). Measurements were made every 24 hours, according to the technique referred to above. The results are shown in Tables I to IV and Text-figs. 1 to 3.

These figures show plainly the continuous decrease of the value of the surface tension when pure serum is heated continuously. The smoothness of the curves indicates that, in most cases, the modifications undergone by the serum between the 1st and 24th hours, and between the 24th and 48th hours, for instance, are quite similar.

¹ du Notty, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707.

² du Notty, P. L., *J. Exp. Med.*, 1922, xxxvi, 115.

Consequently, the destruction of complement which takes place in the first 2 hours does not manifest itself by an important change in the value of the surface tension, or else the curves would show some kind of a break, a maximum or a minimum; moreover, if the change is

TABLE I.

Dog Serum, No. 976 (Text-Fig. 1).
Temperature 23°C.

Time, hrs.....	0	24	48	72	96	120	130
Surface tension, dynes.....	57.0	56.5	56.0	55.0	54.0	53.0	Coagulated.

TABLE II.

Dog Serum, No. 984 (Text-Fig. 1).
Temperature 23°C.

Time, hrs.....	0	24	48	72	96	120	130
Surface tension, dynes.....	56.0	55.0	53.0	51.9	52.5	50.0	Coagulated.

TABLE III.

Dog Serum, No. 998 (Text-Fig. 2).
Temperature 23°C.

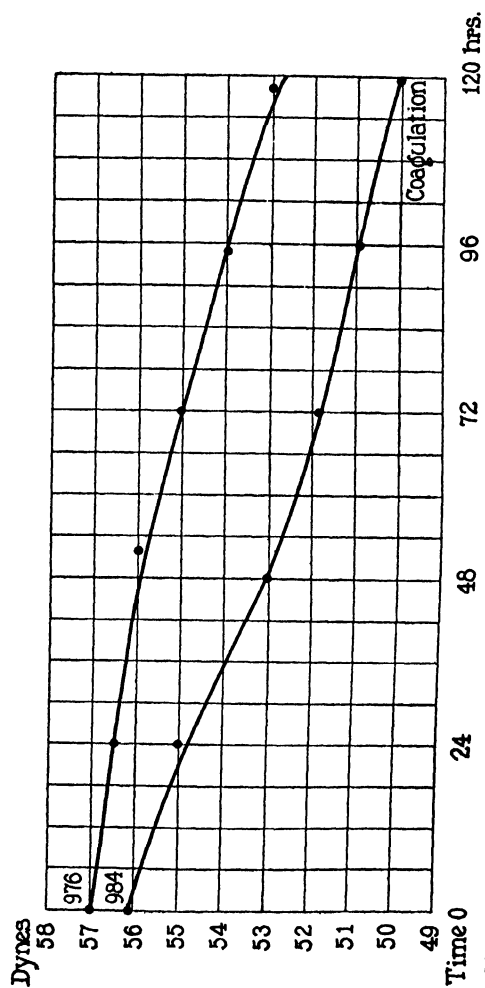
Time, hrs.....	0	24	48	72	96
Surface tension, dynes.....	57.0	57.0	56.0	55.0	54.1

TABLE IV.

Dog Serum, No. 987 (Text-Fig. 3).
Temperature 23°C.

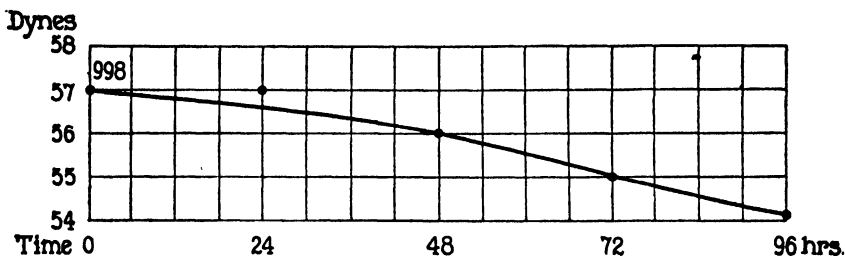
Time, hrs.....	0	24	48	72	96	120	130	168
Surface tension, dynes.....	57.5	54.5	52.3	51.3	50.8	50.5	50.0	49.2

connected in any way with the decrease in surface tension, it is so small in 2 hours that it remains within the range of experimental error. At the end of 120 hours, the reaction is in general almost as marked as at the beginning, the slope of the curve showing no constant tendency to change.



TEXT-FIG. 1. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

As the perturbations due to the destruction of complement may be of such order of magnitude that the presence of highly concentrated colloids would hinder their effect on surface tension, if there is any, experiments were carried out with solutions of serum.



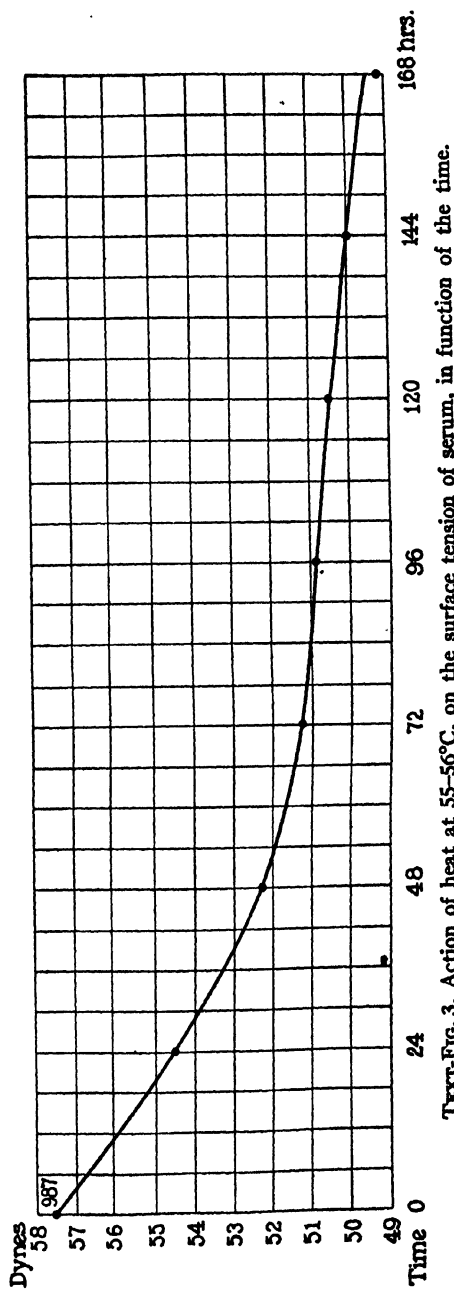
TEXT-FIG. 2. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

II.

Solutions of Serum.

All solutions were made from the same serum, with 0.9 per cent NaCl solution, especially prepared in clean vessels, the surface tension of which was 76.0 dynes $\pm$ 0.2 at 23°C. The solutions were stirred mechanically by an electric motor fitted with a small, propeller-shaped, glass stirrer. The liquid was removed from the container while the stirring was going on and poured into clean test-tubes.

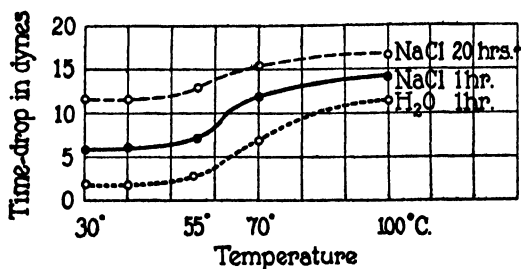
The heating was done in the following way. In most experiments six dilutions were prepared, 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} . About 6 cc. of each dilution were poured into four test-tubes which were prepared at the same time. One was kept at room temperature, one at 55°C. for 2 hours, one at 70°C. for 1 hour, and one at 100°C. for 5 minutes. In order that the same time should elapse between the preparation of the solutions and the measurements, the measurements were begun only 1 hour after the tube at 55°C. had been removed from the incubator, so that approximately 3 hours elapsed between the pouring into the test-tubes and the measurements. Before the liquid was poured into the watch-glasses, it was carefully stirred. Then after 2 hours, another reading was taken. Hence the time required for one set of measurements, at one concentration, was 5 hours. In this case, the time-drop was studied because the initial values, on ac-



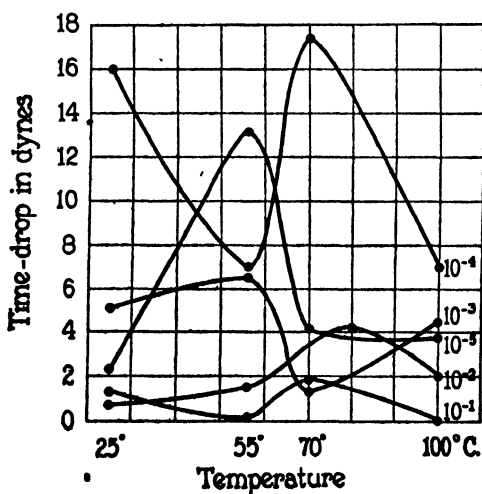
TEXT-FIG. 3. Action of heat at 55-56°C. on the surface tension of serum, in function of the time.

count of the dilution, do not show any marked differences, except when heated at 100°C.

In order to save space, and because the accuracy of the figures does not have any bearing on the general results, charts only will be

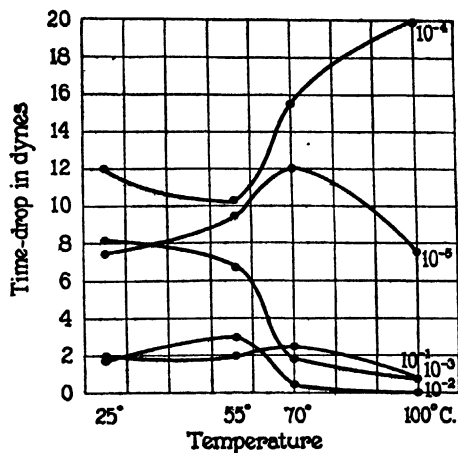


TEXT-FIG. 4. Time-drop of serum solution, concentration 10^{-4} , in saline solution and distilled water (Serum 119).

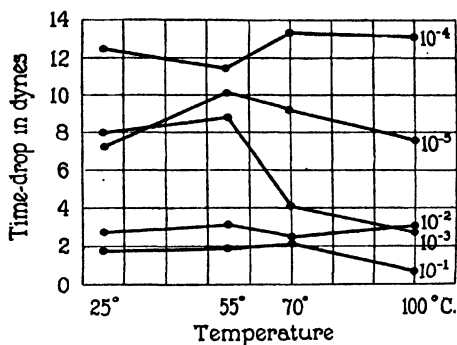


TEXT-FIG. 5. Time-drop in 2 hours, in function of the temperature, at different concentrations (Serum 1696).

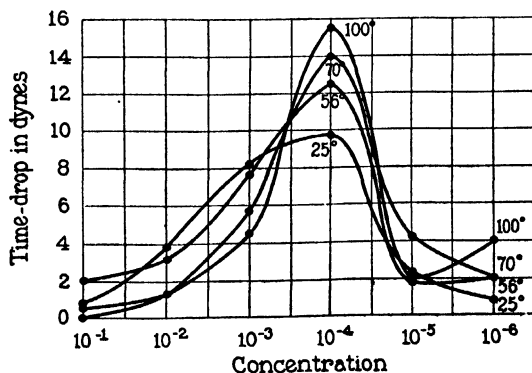
given. A curve expressing the time-drop in 1 and in 20 hours at one concentration is given in Text-fig. 4, with the curve obtained by diluting the serum in distilled water. A fair idea of how puzzling the results seem to be at first glance is given in Text-figs. 5 to 10. No regularity whatsoever is to be found. Unless an hypothesis is made, it is im-



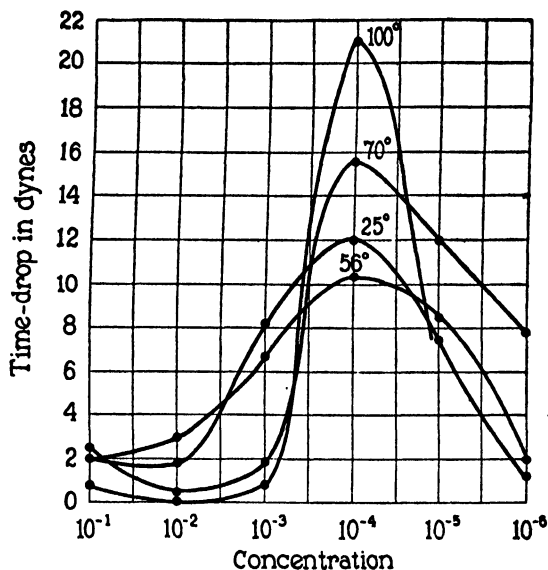
TEXT-FIG. 6. Time-drop in 2 hours, in function of the temperature, at different concentrations (Serum 1711).



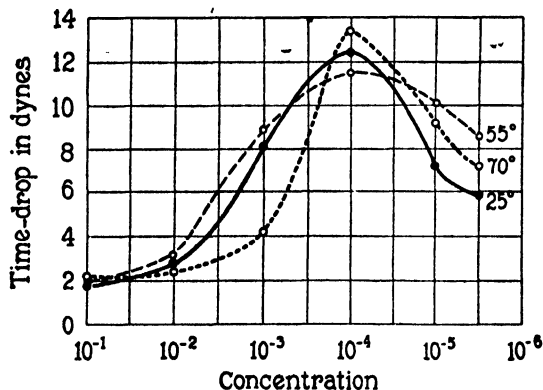
TEXT-FIG. 7. Time-drop in 2 hours, in function of the temperature, at different concentrations. Mean values of ten experiments.



TEXT-FIG. 8. Time-drop in 2 hours, in function of the concentration, at different temperatures (Serum 1802).



TEXT-FIG. 9. Time-drop in 2 hours, in function of the concentration, at different temperatures (Serum 1711).



TEXT-FIG. 10. Time-drop in 2 hours, in function of the concentration, at different temperatures. Mean values of ten experiments.

possible to interpret these results. The mean values of ten of the charts, in other words the algebraic sums of 480 measurements, are represented by Text-figs. 7 and 10.

III.

DISCUSSION.

The interpretation of these results is difficult, and it is not at all certain that it will be successful. However, we shall endeavor to give a possible explanation of the curves, which seem, at first sight, contradictory and devoid of meaning.

One is struck by the fact that the most important changes in the time-drop occur at the temperatures 55° and 70°C. (Text-figs. 5 to 7). These temperatures seem to be critical for the serum, and the fact should have attracted attention even had it not been known that they are also critical from the physiological standpoint. On the other hand, the curves representing the behavior of solutions 10^{-1} and 10^{-2} are parallel in general, and do not present the sharp jumps and drops shown by solutions 10^{-3} , 10^{-4} , and 10^{-5} . Finally, it will be noticed that the dilutions 10^{-3} and 10^{-4} generally act in an opposite way.

The first idea which naturally arises is that the surface equilibrium is more stable on either side of the concentration 10^{-4} , that is 10^{-3} and 10^{-5} , so that around 10^{-4} it reaches some sort of peak instability, and the values may rise or drop suddenly, according to the laws of hazard; namely, for such reasons as actually escape our control and knowledge. These causes, although very slight, may produce considerable changes in either direction simply because, the equilibrium being unstable, the phenomenon has just as much tendency to occur one way as the other.

Table V shows, perhaps more clearly than the charts, that the surface equilibrium at the concentration 10^{-4} seems perfectly unstable, while it is more or less determined in a given direction at all other concentrations. Here, the direction only of the time-drop has been taken into account, whether it increased or decreased, at the critical temperatures. The + sign expresses symbolically that the time-drop rose, the - sign that it decreased. As the values were taken from ten experiments, the coefficients were added to show the percentage of the occurrence of the rise or the drop; and it was admitted that one - sign checks a + sign, so that the figures actually express the number of rises or falls in excess. For example, out of the 10 experiments at a dilution 10^{-1} between 25° and 55°C., 2 showed a decrease and

8 showed an increase of the time-drop. The 2 decreases will be given the $-$ sign, and the 8 increases the $+$ sign. But 2 $-$ counteracts 2 $+$, so that we have only 6 $+$ left. The next entry shows that we had 9 $+$ and 1 $-$, the result being 8 $+$. In other words, the figures could be called equilibrium coefficients, 10 being the maximum characterizing perfect equilibrium, and 0 (5 $+$ and 5 $-$) corresponding to perfect instability.

Table V shows that there is a decided maximum of instability at the concentration 10^{-4} , and a much greater instability on the side of higher dilutions than on the side of lower dilutions. As a matter of fact, the presence of the 3 zeros in the 10^{-4} line may be considered as an accident, on account of the small number of experiments. But

TABLE V.

Increase and Decrease of the Time-Drop of Serum Solutions in 2 Hours When Heated at 55°, 70°, and 100°C.

Concentration.	Between 25° and 55°C.	Between 25° and 70°C.	Between 55° and 70°C.	Between 70° and 100°C.
10^{-1}	6+	10+	8+	10-
10^{-2}	8+	2-	8-	0
10^{-3}	6+	10-	10-	2-
10^{-4}	0	0	0	2+
10^{-5}	2+	2+	0	2-
10^{-6}	2-	2-	10-	2+

the results would have been just as convincing had such coefficients as 2 been found; only 100 experiments would be expected to give a real expression of the state of instability. However, there is no doubt that there is a striking difference, which could only be enhanced by a larger number of data, between the upper part of the table and the lower.

This confirms our preceding hypothesis as to the existence of a monomolecular layer at a concentration around 10^{-4} , geometrically arranged so that the sum of all individual molecular attractions in this layer is minimum, or very nearly so. Hence, any minute change, either in the arrangement of the molecules or else in their field of force, will manifest itself by a relatively considerable rise or decrease in the time-drop, owing to the state of unstable equilibrium of the

film. Therefore, it is in the vicinity of this concentration that the most sudden jumps of the surface tension are to be expected (Text-figs. 8 and 9).

From these conclusions, one fact may be remembered; namely, that if certain physiological modifications of the serum are to have a bearing on surface tension or its changes in function of the time, the possibility of detecting them will be greater at the dilution which enables one to bring forth the slightest alterations in the fields of forces of the molecules or group molecules, that is around 1:10,000.

IV.

Crystallization.

Figs. 1 to 4 show the alterations brought about by heating the solution of serum at 1:10, in the crystallization of sodium chloride. However, the changes are not always identical, especially between 25° and 56°C., and all sera do not allow the sodium chloride to crystallize in the same way. The pictures reproduced here are, nevertheless, frequently observed, and other configurations may be considered as more or less exceptional. The reader is referred to the preceding paper² for another manifestation of the action of heat on solutions of serum.

V.

CONCLUSIONS.

1. The surface tension of pure serum, heated at 55°C., decreases progressively and regularly until the serum coagulates. A drop of 8.2 dynes was observed in 168 hours. The mean drop was 5.7 dynes in 120 hours and 4 dynes in 96 hours.

2. The initial surface tension of solutions of serum at the concentrations 10^{-1} up to 10^{-6} is practically not affected by heat, but the time-drop in 2 hours is modified.

3. Each serum seems to react in its own particular way as far as the time-drop is concerned. However, there is a general tendency for the solution to show an increase of time-drop at the concentrations 10^{-1} , 10^{-2} , 10^{-3} , and 10^{-5} , and a decrease at 10^{-4} , when heated at 55°C.; a decrease of the time-drop at the concentrations 10^{-1} , 10^{-2} , and 10^{-3} , and an increase at 10^{-4} when heated at 100°C.

4. Nevertheless, only the changes observed below 10^{-3} are constant in sign, within 75 or 80 per cent of the cases. The concentration 10^{-4} seems to correspond to a state of greater instability. This confirms the hypothesis of the existence of a monomolecular layer at that concentration, which was assumed on the basis of the existence of a maximum drop at 10^{-4} . Should this be true, and provided the principle of Gibbs could be transformed so as to be applicable to mixed solutions of colloids and crystalloids, an idea of the size, or at least of one of the dimensions of the molecules or group molecules composing the serum could probably be obtained.

5. 1 drop of the solutions at 1:10 evaporated on glass and examined under the microscope shows the marked differences brought about by heating at different temperatures.

A great number of the measurements referred to in this paper were made by my technical assistant, Mr. John Zwick, to whom I wish to express my indebtedness.

EXPLANATION OF PLATES.

PLATE 50.

FIG. 1. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at room temperature. $\times 20$.

PLATE 51.

FIG. 2. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 55°C. for 2 hours. $\times 20$.

PLATE 52.

FIG. 3. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 70°C. for 1 hour. $\times 20$.

PLATE 53.

FIG. 4. Photomicrograph of 1 drop of serum diluted in 1:10 saline solution. Kept at 100°C. for 5 minutes. $\times 20$.

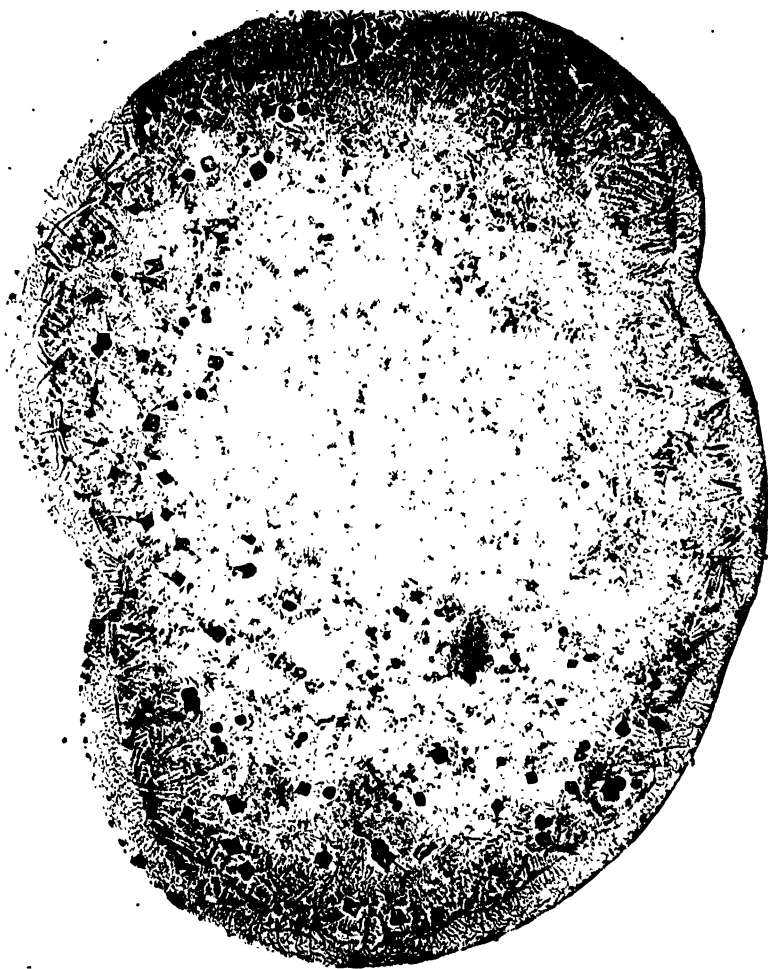


FIG. 1.

(du Noüy: Surface tension of serum. IV.)

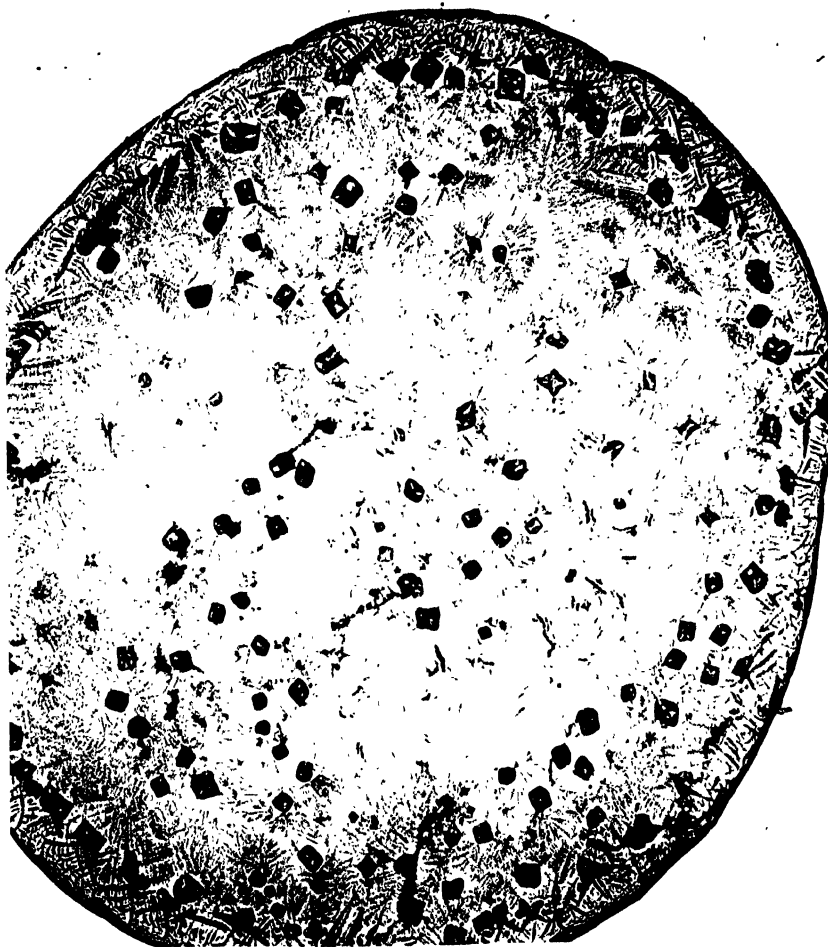


FIG. 2.

(du Noüy: Surface tension of serum. IV.)

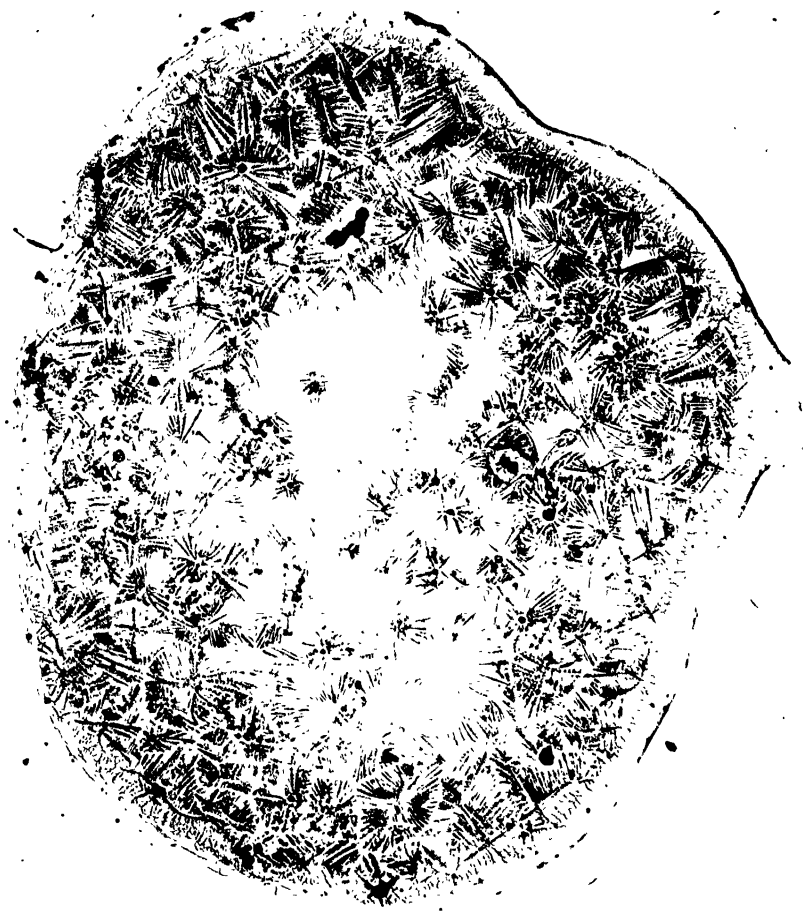


FIG. 3.

(du Notty: Surface tension of serum. IV.)

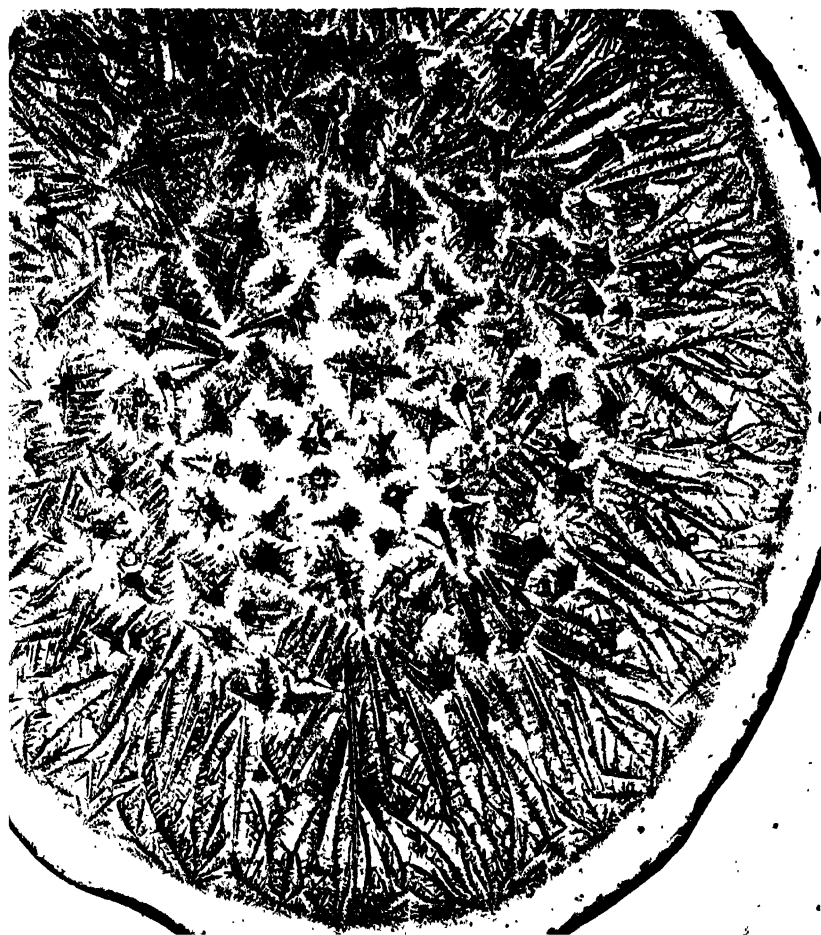


FIG. 4.

(du Nolly: Surface tension of serum. IV.)

LEUCOCYTIC SECRETIONS.

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(Received for publication, June 14, 1922.)

I.

INTRODUCTION.

Leucocytes may be regarded as mobile unicellular glandular bodies which set free their secretions in the humors of the organism. But little is known of the nature and functions of the substances they secrete. In the following experiments, leucocytes were cultivated *in vitro* and a study was made of certain modifications of the medium caused by their secretions.

II.

Technique.

The leucocytes were obtained from hen blood and cultivated in Gabritschewski dishes. The serum was extracted from the culture medium and the variations of its inhibiting action on homologous fibroblasts, and of its lytic action on foreign erythrocytes, were measured.

1. *Preparation of the Leucocytes.*—The blood was taken from young adult chickens which had fasted for 24 hours. The animals were bled from the carotid artery or from one of the veins of the wing, through an oiled cannula or syringe. The blood was received in cold paraffin-coated tubes and centrifuged in ice for 10 minutes. After the plasma had been removed, a few drops of Ringer solution containing a small amount of embryo juice were placed at the surface of the corpuscles and the tubes were allowed to stand at room temperature for about 15 minutes. Then the film of leucocytes, which covered the erythrocytes, became solid, and could be removed to a watch-glass filled with Ringer solution. The film was washed almost free of erythrocytes and cut in small fragments of about equal size.

2. *Preparation of the Medium.*—The medium consisted of chicken plasma and embryo tissue juice. Its composition was generally as follows:

Chicken plasma	5.00 volumes.
Embryonic tissue juice	0.25 volume.
Tyrode solution	2.75 volumes.
Distilled water	2.00 volumes.

The medium was rendered hypotonic on account of the evaporation which takes place in the Gabritschewski dishes. In some cultures, casein was added to the medium. 1 gm. of casein was dissolved in a diluted solution of sodium hydroxide, and dialyzed against tap water for 3 days. Then it was again brought into solution by the addition of a little alkali, and rendered isotonic by a proper amount of NaCl. The pH of the solution was about 7.8. The amount of the solution used in the medium varied from 0.1 to 1 volume of a 1 per cent casein solution; that is, the concentration of casein was from 0.1 to 1 per 1,000.

3. *Preparation of the Cultures.*—The cultures were made on Gabritschewski dishes, the covers of which had been replaced by mica plates. The fragments of leucocytic films were suspended in 5 volumes of Tyrode solution, distilled water, and embryo juice in the concentrations previously described. The same number of fragments was used for each culture. Then 5 volumes of plasma were spread on the mica plate, with the necessary precautions to prevent bacterial contamination from the air. Afterwards, the leucocytic suspension was dropped from a pipette into the plasma, and thoroughly mixed with it. Care was taken to distribute the fragments of leucocyte film evenly. Their number was about thirty to each dish, in order that the colonies might grow freely around each fragment, without being in contact with the edges of the neighboring colonies before 48 hours. Controls were prepared consisting of the medium without leucocytes. Both cultures and their controls were incubated for 48 hours at 38°C. Other cultures and controls were placed in the refrigerator for the same time.

4. *Preparation of the Serum from the Cultures.*—After 48 hours, the cultures were examined. Around each fragment, a large colony of leucocytes had grown. The edges of the colonies were generally in close contact. Microscopic examination showed whether the cells

were living and in active condition, and whether bacterial contamination had occurred. Then the boxes were opened and the coagulum was sliced with fine scissors. The fluid and the fragments of coagulum were centrifuged at high speed for 10 minutes. The supernatant fluid was removed and its H ion concentration ascertained by the technique described by Felton.¹ The serum of the cultures of leucocytes always became slightly acid after 48 hours in the refrigerator. When the colonies had grown actively in the incubator for 48 hours, the acidity of the medium was much more marked. The pH of the serum from the cultures was brought to about that of normal serum by adding a small amount of sodium hydroxide.

5. *Action of Serum on Chicken Fibroblasts and on Foreign Erythrocytes.*—The sera from cultures with and without leucocytes, incubated or kept in the refrigerator, were compared from the standpoint of both their hemolytic effect on sheep or rabbit erythrocytes and their inhibiting power on chicken fibroblasts. The hemolytic action is expressed in the tables by the amount of serum required for a given degree of hemolysis. The action on chicken fibroblasts was ascertained by the rate of growth of a 10 year old strain of fibroblasts in a medium composed of 2.5 volumes of chicken plasma, 5 volumes of serum from leucocyte cultures, 2.2 volumes of Tyrode solution, and 0.3 volume of embryonic juice. In the tables, the rate of growth is indicated by the relative increase of the fragment of fibroblasts in 48 hours. The quotient of the average rate of growth of the experiment by the average rate of growth of the control expresses the action of serum on homologous fibroblasts.

III.

RESULTS.

The experiments consisted in measuring the inhibiting effect on chicken fibroblasts and the hemolytic power on sheep erythrocytes of the serum from media without leucocytes and of the serum from similar media with leucocytes, after the colonies had been allowed to grow for 48 hours. Other experiments were made in order to study the action of temperature on serum and cells. A culture medium without

¹ Felton, L. D., *J. Biol. Chem.*, 1921, xlii, 299.

TABLE I.

Action of Serum from Cultures of Chicken Leucocytes on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					per cent	per cent	per cent
1	1410	1.50	3.00	2.00	0	0	0
2	24858-1	2.65	3.00	1.13	0	0	0
		2.85	3.84				
		2.74	2.56				
3	24858-2	2.19	4.60	1.51	0	0	0
		3.27	4.10				
		2.82	3.44				
4	25171	1.94	3.05	1.60	0	0	0
		2.36	3.65				
		1.86	3.17				
5	25213	2.53	3.97	1.57	0	0	0
		2.94	4.46				
		2.66	4.28				
		3.40	5.43				
6	25391	2.77	3.90	1.45	0	0	0
		2.40	3.35				
		2.02	3.64				
		3.28	4.00				
7	28616	2.59	4.57	1.71	0	0	0
		3.00	4.94				
		2.57	4.16				
8	28801	3.07	5.41	1.56	0	0	0
		3.19	4.37				
9	28684	1.62	3.40	2.00	70	0	40
		2.13	4.09		50	0	20
					30	0	5
10	28641	2.66	4.57	1.37	70	0	45
		4.13	4.29		50	0	25
					30	0	5

TABLE I—*Concluded.*

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
11	28770	6.18	7.00	1.70	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
		3.38	7.68		70	0	0
12	29263	4.41	9.07	2.18	80	7.00	10
		2.57	6.30		40	5.00	10
		2.60	5.29		20	2.50	4
13	28801	3.07	5.41	1.56	70	0	0
		3.19	4.37				
14	28886	2.38	1.70	1.10	70	0	40
		3.10	4.66		50	0	30
					30	0	8
15	28926	0	0		80	5.00	40
					60	2.50	20
					20	2.50	5
					10	2.50	2.50
16	29019	0	0		80	10.00	20
					60	8.00	15
					40	5.00	8
					20	2.50	3
17	29162	1.21	6.77	3.33	80	0	0
		3.20	5.15				
		2.26	6.38				
18	29400	0	0		80	60.00	75
					60	50.00	55
					40	30.00	30
19	29059	1.87	4.14	1.98	80	0	0
		3.86	6.70				
		3.38	6.80				

leucocytes kept in the refrigerator for 48 hours was compared with a similar medium incubated for the same length of time. The sera from culture media with and without leucocytes, kept in the refrigerator instead of being incubated, were also compared.

1. *Serum from Media Incubated for 48 Hours, with and without Leucocytes.*—In every case, the colonies grew actively and were still living after 48 hours. In seventeen experiments (Table I), the serum from the media with leucocytes was more favorable to the activity of fibroblasts. The quotient of the rate of growth in serum from leucocyte cultures divided by the rate of growth in serum from media without leucocytes averaged 1.73.

TABLE II.

Action of Serum from Culture Media, Incubated and Refrigerated, on Homologous Fibroblasts and Foreign Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from culture media.		Ratio, $\frac{E}{C}$.	Hemolytic action on foreign erythrocytes.		
		Incubated (C).	Refrigerated (E).		Amount of serum.	Amount of hemolysis by serum from culture media.	
						Incubated.	Refrigerated.
1*	29094	1.25	6.53	4.49	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
		1.68	6.33		80	8	80
					20	5	50
					10	0	25
2†	29339	1.04	2.38	3.20	70	15	80
		0.87	3.73		50	8	80
		1.34	4.10		30	3	70
					80	55	75
3†	29436	0	0		40	40	70
				10	10	20	

* In Experiment 1, sheep erythrocytes were used.

† In Experiments 2 and 3, rabbit erythrocytes were used.

The hemolytic power of the serum on sheep or rabbit erythrocytes was measured in eleven experiments. In four cases (Table I), both sera were inactive. In seven cases (Table I), the sera from cultures without leucocytes were inactive, while the sera from cultures with leucocytes were hemolytic for sheep or rabbit corpuscles. After the serum had been heated at 56°C., its hemolytic action disappeared completely.

(a) *Serum from Culture Media without Leucocytes, Incubated and Refrigerated.*—The effect of heat at 38°C. for 48 hours on media without leucocytes was examined (Table II). The natural hemolytic action of the serum on sheep or rabbit corpuscles decreased after the medium had been incubated, while it remained normal when the medium was kept in the refrigerator. At the same time, the serum from the incubated medium became much less favorable to the growth of chicken fibroblasts (Table II).

TABLE III.

Action of Serum from Cultures of Chicken Leucocytes, Refrigerated, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
		Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
						Without leucocytes.	With leucocytes.
					<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	28684	1.33	2.14	1.31	70	40	60
		1.92	2.03		50	20	20
		1.91	2.50		30	5	10
2	28641	5.20	3.83	0.73	70	70	80
					30	10	30
3	28770	4.83	4.82	0.99	50	100	100
		7.93	7.85		30	80	35
4	29263	4.06	4.55	1.91	60	35	35
		2.59	5.16		40	8	20
		2.85	7.47		20	2.5	10
					10	2.5	5

(b) *Serum from Culture Media with and without Leucocytes, Refrigerated for 48 Hours.*—In every experiment (Table III), the hemolytic power of the serum from media without leucocytes remained normal, while that from media with leucocytes was slightly increased. At the same time, there was no marked difference in the average rates of growth of fibroblasts in both sera.

2. *Serum from Media with and without Leucocytes, to Which a Foreign Protein Was Added.*—When 1 per 1,000 casein was added to a culture medium containing no leucocytes, the serum became more inhibiting

for the growth of fibroblasts. It was less toxic when the medium contained only 0.1 per 1,000 casein. A comparison of both sera from cultures of leucocytes with and without 1 per 1,000 casein showed that the presence of casein increased the amount of leucocytic secretion. The juice of the cultures containing casein was always a better medium for homologous fibroblasts than that of the cultures without casein, in spite of the toxic action of the casein (Table IV).

TABLE IV.

Action of Serum from Cultures of Chicken Leucocytes, without and with Casein, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from leucocytic cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
						Amount of serum.	Amount of hemolysis by serum from leucocytic cultures.	
			Without casein (C).	With casein (E).			Without casein.	With casein.
						per cent	per cent	per cent
1	28801	1 per 1,000	4.49	8.80	1.71	70	0	0
			4.16	7.70				
			7.90	10.50				
2	28886	1 " 1,000	4.81	5.35	1.34	70	40	40
			2.75	4.23		50	30	30
						30	8	8
3	1410	Dog serum.	1.00	2.50	2.50	0	0	0
4	28988	0.1 per 1,000	2.05	2.10	1.03	80	0	0
			1.54	1.32				
			1.81	2.21				
5	29059	0.1 " 1,000	6.71	6.70	1.17	80	0	0
			5.31	7.25				

The quotient of the rates of growth of fibroblasts in serum from casein media with and without leucocytes was higher than the quotient of the rates of growth of similar cultures containing no casein (Table V). There was little or no difference in the action of the sera when the concentration of the casein in the culture media was 0.1 per 1,000 (Table V).

TABLE V.

Action of Serum from Cultures Containing Casein, without and with Chicken Leucocytes, on Homologous Fibroblasts and Sheep Erythrocytes.

Experiment No.	Culture No.	Casein concentration in medium.	Rate of growth in serum from cultures.		Ratio, $\frac{E}{C}$.	Hemolytic action on sheep erythrocytes.		
			Without leucocytes (C).	With leucocytes (E).		Amount of serum.	Amount of hemolysis by serum from cultures.	
							Without leucocytes.	With leucocytes.
						<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	28801	1 per 1,000	1.00	4.04	4.40	70	0	0
			0.95	4.84				
			1.13	4.63				
2	28886	1 " 1,000	1.00	1.94	2.03	0	0	0
			1.00	2.00				
			1.14	2.10				
3	28926	0.5 per 1,000	0	0		80	5	50
						60	3.5	30
						40	2.5	15
4	29019	0.5 " 1,000	0	0		80	10	25
						60	8	20
						20	2.5	5
5	29162	0.5 " 1,000	2.88	10.00	4.39	80	0	0
			1.25	5.76				
			1.69	8.66				
6	29400	0.5 " 1,000	0	0		80	60	75
						60	50	65
						40	30	40
7	29059	0.1 " 1,000	6.80	12.77	2.06	80	0	0
			4.81	10.35				
			2.61	5.68				
8	29435	0.5 "	0.65	2.93	4.34	0	0	0
			1.19	4.90				

No increase of the lytic action on foreign erythrocytes of the serum from culture media containing casein was observed.

IV.

DISCUSSION, SUMMARY, AND CONCLUSIONS.

In the present condition of the technique of cultivation of tissues, the only possible way of studying leucocytic secretions was to grow colonies of leucocytes in a medium of known properties and to examine the modifications of these properties under the influence of the living cells. The method was far from perfect, because the secretions were mixed with serum and accumulated for 48 hours in a medium where they probably underwent partial destruction. But an approximate idea of certain of the qualities of the secretions, although not of their quantity, could be derived from the experiments. In the fluids extracted from the cultures, we attempted to detect the presence of the leucocytic secretions through their physiological effects on homologous and foreign cells. Two kinds of substances were sought, those which act on homologous cells, and those which destroy foreign erythrocytes.

The secretion by leucocytes of substances necessary to the nutrition of other cells was considered as probable long ago. Renault² thought that the main function of the white blood corpuscles was to bring to the fixed cells of the tissues the food material which they need. While the existence of physiological relations between leucocytes and tissue cells could be considered as almost certain, their nature had remained practically unknown. It was probable, however, that the substances secreted by leucocytes were analogous to the growth-activating and unstable substances which are found in embryonic tissues, leucocytes, and certain adult tissues.³ When connective tissue was aseptically inflamed, or when an aseptic peritoneal exudate contained many leucocytes, aqueous extracts of both connective tissue and peritoneal exudate were found to have acquired the power of stimulating cell proliferation.⁴ These experiments showed that leucocytes could bring to the tissues some activating substances. But it remained to be ascertained whether leucocytes, while they are alive, could secrete similar substances either spontaneously or under the stimulus of a foreign factor.

² Renault, J., *Traité d'histologie pratique*, Paris, 1893, i, pt. 2, 968.

³ Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; xviii, 287.

⁴ Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.

Leucocytes are supposed to be, as is well known, the origin of the substances which protect the organism against infection.⁵ Although the problem of the origin of alexin and antibodies has been investigated by many experimenters, it is not yet completely solved. It was of interest, therefore, to ascertain whether leucocytic secretions could increase the natural hemolytic effect of hen serum on sheep or rabbit erythrocytes, and whether these secretions would become more active under the influence of a foreign protein. The substances which destroy foreign cells are not necessarily different from those which act on homologous cells. The word substance is used for simplicity of description and may be taken as meaning only a given property of an unknown substrate.

A comparison was made of certain properties of sera extracted after 48 hours incubation from media containing leucocytes and from media containing no leucocytes. The serum from the leucocytic cultures was always found to be more favorable to the growth of homologous fibroblasts than the serum from the culture media incubated without leucocytes. The natural hemolytic power of the serum on sheep erythrocytes was found to be increased in about 50 per cent of the experiments.

In other experiments, we found that when two culture media free of cells were placed, one in an incubator at $+38^{\circ}\text{C}$. and the other in a refrigerator at $+5^{\circ}\text{C}$. for 48 hours, the serum from the incubated medium partly lost its hemolytic action on sheep or rabbit erythrocytes, while that from the refrigerated medium remained normal; at the same time, the inhibiting action of the incubated medium on homologous fibroblasts had increased very much. This effect of incubation indicates that certain unstable constituents of serum are destroyed by heat. Then the changes found in the properties of the serum from cultures of leucocytes are due to the fraction of the activating substances which has not been destroyed by incubation at 38°C . A quantitative study of the secretions is, therefore, impossible with the present technique, which can furnish only qualitative indications about the substances set free by the leucocytes.

⁵ Metchnikoff, E., *Immunity in infective diseases*, translated by Binnie, F. G., Cambridge, 1905.

We have ascertained also whether a medium containing leucocytes and kept in the refrigerator undergoes any change under the influence of the cells while they are in a condition of latent life. Gabritschewski dishes with and without leucocytes were placed in a refrigerator at a temperature of about $+5^{\circ}\text{C}$. After 48 hours, the hemolytic power on sheep erythrocytes of the serum from the leucocytic cultures had increased slightly and its inhibiting action on the growth of homologous fibroblasts had decreased. Then certain substances favorable to the growth of homologous cells and toxic for heterologous cells were diffused by the leucocytes into their medium. But the action of these substances was weaker than in the case of the cultures kept in the incubator. This experiment showed that leucocytes under certain conditions diffuse alexin or natural hemolysins which originate from them at the same time as the substances which activate homologous cells. In other experiments, although leucocytes were frozen at -10°C ., treated with distilled water, or extracted with saline solution, they did not yield any hemolysin.

To summarize: Leucocytes, cultivated in plasma, always secreted substances which increased the rate of growth of homologous cells. Less frequently, they set free substances which hemolyzed foreign erythrocytes.

The growth-promoting substances are analogous to those contained in embryonic tissues, and probably represent some of the foodstuffs brought to fixed tissue cells by leucocytes. They may possess the function of rejuvenating cells which have ceased to multiply when the cicatrization of a wound or the repair of a fracture requires a resumption of tissue activity.⁴ According to this hypothesis, the leucocytes brought to the surface of a wound by the process of inflammation would not only oppose bacterial invasion, but also bring to the tissues the material necessary to cell multiplication. It seems that in some cases regeneration is started by substances brought to the tissues by other cells. Loeb thinks that in *Tubularia*, when endodermic cells gather at the end where a new polyp is about to be formed, the substances given off by these cells are responsible for polyp formation.⁶ There may be

⁶ Loeb, J., *The organism as a whole from a physicochemical viewpoint*, New York and London, 1916, 170.

an analogy between this phenomenon and the secretion by leucocytes of growth-activating substances at the surface of a wound.

If we assume that leucocytes *in vivo* set free their secretions in the blood stream, certain variations of the growth-inhibiting action of normal serum can be better understood. The rate of proliferation of homologous fibroblasts is much slower in the serum of an old chicken than in that of a young one.⁷ When the serum is heated at 56° and 70°C. for $\frac{1}{2}$ hour, it becomes still more inhibiting.⁸ A substance favorable to cell activity has disappeared. It is therefore permissible to suppose that the growth-inhibiting power of serum and its variations are due to the antagonistic action of two substances, one growth-promoting and thermolabile, and the other growth-inhibiting and thermostable, the activating substance being always weaker in its effect than the inhibiting one. We know that activating substances can be extracted from embryonic tissue, from muscle and gland tissues, and from leucocytes of the adult animal, and that they are thermolabile and very unstable. Leucocytic secretions seem to have some of the properties of leucocytic extracts. It is probable that the activating substances which disappear from the heated serum are secreted by leucocytes and other cells. An increase of these secretions, then, would diminish the inhibiting action of serum on homologous fibroblasts. On the contrary, a decrease of the secretions in the serum would increase its inhibiting effect on homologous cells. The strong inhibiting action of serum in old age would be due partly to a reduction in the amount and activity of the substances secreted by leucocytes and tissue cells in the humors of the organism.

Leucocytes also secreted *in vitro* substances which were toxic for foreign cells. Although the results were not constant, the serum appeared to become slightly more hemolytic for sheep or rabbit erythrocytes, under the influence of the leucocytes. The hemolysis of rabbit corpuscles by hen serum is due, according to Hyde,⁹ to a complex sensitizer alexin, and not merely to alexin, as Bordet thought.¹⁰

⁷ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599.

⁸ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 647.

⁹ Hyde, R. R., *Am. J. Hyg.*, 1921, i, 358.

¹⁰ Bordet, J., quoted by Hyde,⁹ p. 358.

When a foreign protein was added to the culture medium, the leucocytic secretions increased, as was shown by the action on homologous fibroblasts of sera taken from cultures of leucocytes with and without casein. The presence in the medium of the cultures of leucocytes of only 0.1 per 1,000 casein did not markedly modify the action of their serum on the proliferation of fibroblasts. When the concentration of casein in the leucocyte cultures reached 1 per 1,000, the growth of chicken fibroblasts in the serum extracted from the Gabritschewski dishes became more rapid. But there was no parallel increase of the hemolytic action of the serum upon sheep erythrocytes.

We found that chicken serum containing 0.1 per 1,000 casein was barely toxic for homologous fibroblasts, while it became markedly inhibiting when the casein concentration reached 1 per 1,000. Probably, there is a relation between the toxicity of the medium, the increase of leucocytic secretions, and the time of the increase. The change brought about by casein in the equilibrium of the system composed of the cells and their medium determines the secretion by the leucocytes of substances which increase the activity of homologous cells and oppose the inhibiting effect of the foreign proteins. This reaction of the leucocytes is immediate, and may represent the first defense of the organism against a factor which disturbs its equilibrium. Possibly it differs from the specific cell reaction which leads to the production of antibodies. It is known that antibodies develop more slowly. Hemolysins were detected in cultures of bone marrow 4 days after the addition of antigen.¹¹ The immunization of fibroblasts against foreign proteins has been shown by Fischer¹² to begin after 4 days. If leucocytes behave in the organism as they do *in vitro*, we may assume that before the appearance of antibodies, they respond to the presence of an antigen by setting free growth-promoting substances and possibly alexin. This immediate reaction of the leucocytes against a disturbing factor, and the resulting production of substances which increase the activity of homologous cells, might be partly responsible for the results observed in the treatment of certain diseases by the injection of foreign proteins.

¹¹ Carrel, A., and Ingebrigtsen, R., *J. Exp. Med.*, 1912, xv, 287.

¹² Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 535.

It may be concluded that, under the conditions of the experiments:

1. The serum obtained from cultures of leucocytes is less inhibiting for homologous fibroblasts than the serum from media without leucocytes. In some experiments, its hemolytic action on sheep or rabbit erythrocytes is also increased.

2. The addition of casein to leucocytic cultures brings about a decrease in the inhibiting effect of the serum on homologous fibroblasts.

3. The increase in the activity of homologous fibroblasts in serum obtained from leucocytic cultures is probably due to growth-promoting substances secreted by the leucocytes. The presence of a foreign protein under certain conditions determines a more abundant leucocytic secretion.

ON THE NATURE OF THE FORCES WHICH DETERMINE THE STABILITY OF AQUEOUS SOLUTIONS OF GELATIN AT THE ISOELECTRIC POINT.

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The stability of solutions or suspensions may be guaranteed by two kinds of forces,¹ namely, first, the forces of attraction between the molecules of the solvent and the molecules of the solute, and second, the forces of repulsion between the micellae. When the forces of attraction between the molecules of the solvent and the molecules of the solute (which may be secondary valency forces) are greater than the forces of attraction between the molecules of the solute for each other the solution will be stable. This type of force acts in the general case of solutions of crystalloids.

When the forces of attraction between the molecules of solute and solvent are weak, the molecules of the solute upon colliding will adhere to each other and aggregates will be formed. This aggregate formation will lead to a flocculation or coagulation of the whole mass unless new forces originate in the small nascent aggregates (or micellae which prevent their coalescence into larger aggregates. These forces may be electrical charges whereby the nascent micellae repel each other. The writer has investigated the origin of these charges in the case of protein micellae and has found that they are essentially due to the occlusion of protein ions in the micellae, the protein ions being unable to diffuse out of the gel, while the latter is permeable to the crystalloidal ions in solution. In this case an unequal distribution of the crystalloidal ions will occur inside the solid protein particles and the bulk of the solution according to Donnan's theory of membrane equilibria. These equilibria lead to the establishment of a potential difference between each particle and the surrounding solu-

¹ LOEB, J., and LOEB, R. F., *J. Gen. Physiol.*, 1921-22, IV, 187. LOEB, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922, p. 243.

tion. The writer has made measurements of these P.D. and has shown that they can be calculated with a great degree of accuracy from the difference in the concentration of crystalloidal ions (e.g., hydrogen ions) inside and outside the particle.² This P.D. guarantees the stability of the suspension inasmuch as it prevents the coalescence of the small micellae into larger masses. It is obvious that this type of force can only come into play when the forces of attraction between the individual protein particles and water are too small to prevent the formation of micellae.

When proteins are at the isoelectric point they are absolutely or nearly non-ionized. Since without ionization of protein no Donnan equilibrium can be established between micellae and surrounding solution, the stability of solutions of proteins at the isoelectric point cannot be ascribed to the existence of electrical charges of the micellae or of the protein molecules. Yet it is a fact that certain proteins, e.g., crystalline egg albumin, form very stable solutions at the isoelectric point. It must, therefore, be inferred that isoelectric albumin is kept in solution by the forces active in ordinary solubility; or in other words, the forces of attraction between the molecules of egg albumin and water must be considerably greater than the forces of attraction between the molecules of crystalline egg albumin themselves. This is true as long as the temperature and concentration of the isoelectric egg albumin are sufficiently low.

The case is somewhat different for isoelectric gelatin which at room temperature is sparingly soluble in water of the p_H of its isoelectric point, i.e., p_H 4.7. A 1 per cent solution of isoelectric gelatin of p_H 4.7 becomes opaque on standing at 20°C., and the suspended particles gradually settle to the bottom of the test-tube provided that the solution does not set to a gel. When, however, a salt is added to such a solution (taking care that the p_H remains at 4.7), the gelatin remains clear at room temperature. In this case it might be assumed that the isoelectric gelatin is not held in solution by the forces active in ordinary solubility but by electrical charges of micellae. The clearing of the solution upon the addition of a salt might in this

² LOEB, J., *J. Gen. Physiol.*, 1920-21, III, 667; 1921-22, IV, 351, 463. Proteins and the theory of colloidal behavior, New York and London, 1922, p. 120.

case be ascribed to the preferential adsorption of one kind of ion of the salt (especially that of higher valency) whereby the charge of the ion is transferred to the particle. As a consequence of this charge the nascent micellae might repel each other to such an extent that the solution remains clear. Yet it can be shown that salts increase the solubility of isoelectric gelatin not by conferring an electrical charge upon particles of gelatin, but in some other way, in all probability by increasing the forces of attraction between the molecules of protein and those of the solvent (*i.e.*, the salt solution).

When less than 1 gm. of isoelectric gelatin is dissolved in 100 cc. of water, the non-dissolved quantity of the gelatin and the turbidity are correspondingly diminished; when only 0.1 gm. is dissolved in 100 cc. of water, the solution remains clear. By thus dissolving 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 gm. of isoelectric gelatin in 100 cc. of water of p_H 4.7, we can make a scale of turbidity which can be used to compare the relative effect of salts on the solubility of isoelectric gelatin at room temperature.

Doses of 1 gm. of isoelectric gelatin were added to 100 cc. of different salts of various concentrations at p_H 4.7, and 10 cc. of the solution were kept in test-tubes at low temperature (about 2° C.) for 24 hours. The test-tubes were then brought back into the room and after 2 hours their relative degree of turbidity was compared with the turbidity scale of solutions of different concentrations of isoelectric gelatin in water without salt, as stated above, which had also been kept at 2° for 24 hours. To make use of the scale we will call the degree of turbidity of a 0.1 gm solution of isoelectric gelatin in water 1, that of 0.2 gm. gelatin in water, 2, and so on. Table I gives the results.

The upper horizontal column gives the molecular concentrations of the salt solutions, and the other figures indicate the degree of turbidity, according to the scale. Thus taking the values for $NaCl$ the table gives 2.0 for $M/2 NaCl$, which means that the degree of turbidity of a 1 per cent solution of isoelectric gelatin in $M/2 NaCl$ was equal to that of a solution of 0.2 gm. gelatin in water without salt; while the turbidity value 5.5 for $M/32 NaCl$ signifies that the degree of turbidity of 1 gm. isoelectric gelatin in 100 cc. of $M/32 NaCl$ of p_H 4.7 was between that of a solution of 0.5 and 0.6 gm. of isoelectric gelatin in water without salt.

It is obvious from the table that the solvent power of water of p_H 4.7 for isoelectric gelatin is increased by salt and the more so the greater the valency of one of the ions. If we take as a standard of comparison that concentration of salt which is required to reduce the turbidity of a 1 per cent solution of 1 gm. of isoelectric gelatin in 100 cc. solution of salt to that of a solution of 0.3 gm. of isoelectric gelatin in 100 cc. H_2O without salt, we find the following concentrations:

TABLE I.
Influence of Salts on Solubility of Isoelectric Gelatin in Solutions of pH 4.7.

Concentration.	1 M	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	M/8,192	M/16,384
NaCl.....	2.0	2.0	2.5	3.0	4.0	5.5	7.0	8.5	9.0	9.5	10.0	10.0			
LiCl.....		2.0	2.5	3.0	4.0	5.5	7.0	8.5	9.0	9.5	10.0	10.0	10.0		
KCl.....		2.0	2.0	2.5	3.5	4.5	6.5	8.5	9.0	10.0	10.0	10.0			
CaCl ₂		1.0	1.5	2.0	2.5	2.5	3.5	4.5	6.0	8.0	8.5	9.0			
MgCl ₂					2.0	2.5	3.0	3.5	5.0	6.0	7.0	8.5	9.0	9.0	
SrCl ₂					2.0	2.5	3.0	4.0	5.5	6.0	7.0	8.0	8.5	9.0	10.0
BaCl ₂						3.5	3.5	4.5	6.0	7.0	8.5	9.0	9.5	10.0	10.0
MgSO ₄				2.0	2.0	2.5	3.0	4.0	6.0	7.5	9.0	10.0	10.0	10.0	10.0
Na ₂ SO ₄		5.5	2.5	2.+	2.+	2.5	3.0	5.0	7.0	8.0	9.0	10.0	10.0		
LiCl ₃					2.0	1.5	1.0	1.0	1.0—	1.0—	1.0	2.0	3.0	7.5	0.0
CaCl ₃									Almost like water			1.0	1.0	2.0	
Na ₄ Fe(CN) ₆									All clear like water					2.5	

About M/8 or slightly less for $LiCl$, $NaCl$, and KCl .

" M/64 for $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, Na_2SO_4 , $MgSO_4$.

" M/4,096 or less for $LaCl_3$, $CeCl_3$, and $Na_4Fe(CN)_6$.

Attention is called to the fact shown in Table I that Na_2SO_4 diminishes the solubility of isoelectric gelatin in water when the concentration of Na_2SO_4 is M/2. This is in agreement with the observation that sulfates are more efficient than chlorides for the salting out of gelatin from aqueous solution.

These results were supplemented by measurements of the rate at which powdered particles of isoelectric gelatin are dissolved in water of p_H 4.7. Powdered gelatin of not too small a size of grain (going through the meshes of sieve 30 but not through sieve 60) was rendered isoelectric in the way described in the writer's previous publications, and about 0.8 gm. was put into 100 cc. of water or a series of salt solutions varying in concentration, but all having the same p_H , namely 4.7. The suspensions of the powdered gelatin were frequently stirred and the time required to practically completely dissolve all the grains of powdered gelatin in suspension was measured at 35° C. The time required to dissolve the gelatin without salt was a little over 2 hours. Table II, giving the influence of salts on the solution time, shows that the time required for solution is diminished by salts and the more rapidly the higher the valency of one of their ions.

TABLE II.

Time in Minutes Required to Dissolve 0.8 Gm. of Powdered Isoelectric Gelatin at 35° C.

Concentration.	M/256.	M/512.	M/1,024.
$LiCl$	57	70	76
$NaCl$	49	66	75
KCl	56	70	80
$MgCl_2$	32	40	61
$CaCl_2$	32	40	62
$BaCl_2$	31	46	66
$CeCl_3$	26	35	44
$LaCl_3$	23		
Na_2SO_4	34	46	60
$Na_4Fe(CN)_6$	24	32	41

Thus while without salt it requires about 120 minutes to dissolve the isoelectric gelatin, the time is reduced to about 67 minutes in M/512 $LiCl$, $NaCl$, or KCl , to about 40 to 45 minutes in M/512 $MgCl_2$, $CaCl_2$, $BaCl_2$, or Na_2SO_4 , and to about 33 minutes in M/512 $CeCl_3$ or $Na_4Fe(CN)_6$. These observations are in harmony with the idea that we are dealing here with a phenomenon of ordinary solubility, inasmuch as the molecules of the salt increase the force of attraction between molecules of gelatin and molecules of solvent.

There exists an excellent criterion which permits us to decide whether the stability of particles in a solution is due to a P.D. between particles and solution or whether it is due to some other cause, *e.g.*, the forces of ordinary solubility. Whenever the stability of a solution (or suspension) is due to a P.D. between the particle and the surrounding solution a low concentration (about $M/8$ or less) of a salt like $CaCl_2$ or $NaCl$ suffices to annihilate the P.D. If we find that a solution or suspension cannot be precipitated even by high concentrations of $CaCl_2$ or $NaCl$, etc., we may be certain that the forces determining the stability of the solution are not the P.D. between the particles and the solution.

The writer has recently shown that salts with trivalent cations, *e.g.*, $LaCl_3$, cause a P.D. between solid isoelectric gelatin and surrounding solution (of p_H 4.7), the gelatin assuming a positive charge,³ and he has given facts suggesting that in this case the gelatin forms an ionizable salt in which the cation is gelatin- La and the anion Cl . Now we have seen in this paper that little $LaCl_3$ suffices to make a 1 per cent solution of isoelectric gelatin completely soluble. If this effect were due to the production of this P.D. between the protein particles and the solution, the gelatin should be precipitated by that low concentration of $CaCl_2$ or $NaCl$ which suffices to annihilate the P.D., *e.g.*, $M/8$. We have seen that 1 per cent solutions of isoelectric gelatin remain permanently as clear as water if they are made up in $M/1,024$ or $M/512$ or $M/256$ or $M/128$ $LaCl_3$. Yet it is impossible to precipitate any of these 1 per cent solutions of gelatin in $M/128$, or $M/256$, or $M/512$, etc., $LaCl_3$ with any concentration of $CaCl_2$ up to $2\frac{1}{2}$ M (higher concentrations were not tried). Samples of 10 cc. of a 1 per cent solution of isoelectric gelatin made up in $M/1,024$ and $M/512$ solutions of $LaCl_3$ could not be precipitated even with 50 cc. of 5 M $NaCl$. About 11 cc. of 2M $(NH_4)_2SO_4$ were required to precipitate isoelectric gelatin in 10 cc. of a 1 per cent solution made up in $M/1,024$ to $M/64$ $LaCl_3$. These experiments contradict the idea that the precipitation was caused by an annihilation of the electrical charges of the gelatin particles, and we may conclude that the increase in the solubility of isoelectric gelatin in water by salts is not due to electrical charges

³ LOEB, J., *J. Gen. Physiol.*, 1921-22, IV, 463, 741. Proteins and the theory of colloidal behavior, New York and London, 1922, pp. 164-165.

conferred upon the protein particles by the ions of the salt. This conclusion is supported by the fact that although according to the writer's measurements salts like Na_2SO_4 , $CaCl_2$, or $NaCl$ do not cause any P.D. between isoelectric gelatin and solution, they increase the solubility of isoelectric gelatin in water.

When traces of acid or of alkali are added to a 1 per cent solution of isoelectric gelatin so that the p_H is either greater than 4.8 or less than 4.6, the solution also becomes clear. In this case some of the gelatin becomes ionized and the establishment of a Donnan equilibrium between particles and surrounding solution becomes theoretically possible. Yet it can be shown that the addition of acid or alkali clears the solution merely by increasing the ordinary solubility of gelatin in water and not by giving rise to a P.D. between micellae of gelatin and solution. Not only is a comparatively low concentration of salt sufficient to annihilate a P.D. due to a Donnan equilibrium, but the annihilation of the P.D. is due to that ion of the salt which has the opposite sign of charge to that of the particle itself, and the efficiency of this ion increases with its valency.

If we add alkali, *e.g.*, $NaOH$, to isoelectric gelatin, Na gelatinate is formed and the gelatin ion has a negative charge. If the clearing of a solution of Na gelatinate were due to an annihilation of electrical charges of micellae by the precipitating salt, a solution of $CaCl_2$, should be much more efficient than a solution of Na_2SO_4 for the precipitation of Na gelatinate from an aqueous solution. As a matter of fact, however, a solution of Na gelatinate can be precipitated only by high concentrations of salts and more easily by Na_2SO_4 than by $CaCl_2$ or $NaCl$. This leaves no doubt that the addition of alkali or acid to isoelectric gelatin increases its solubility in water not through the formation of electrically charged particles but by increasing the forces of attraction between the molecules of the gelatin and the molecules of water (or acid in water or alkali in water).

The idea that the forces which keep proteins in solution must under any circumstances be different from the forces which keep crystalloids in solution was a purely arbitrary and gratuitous assumption made at the time when it was also believed that proteins did not react stoichiometrically with acids and alkalies. The proof of the stoichiometrical character of the reactions of proteins with acids and alkalies made it

possible to explain the colloidal behavior of protein solutions mathematically and quantitatively on the basis of Donnan's theory of membrane equilibria.⁴ Since the Donnan equilibrium depends only on the condition that one type of ions is prevented from diffusing while the other ions are able to diffuse (regardless of whether the non-diffusible ion is termed colloid or crystalloid) there is no *a priori* reason left to assume that the forces which keep proteins in solution must be under all circumstances different from the forces which keep crystalloids in solution.

⁴ LOEB, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

E STABILITY OF BACTERIAL SUSPENSIONS.

IV. THE COMBINATION OF ANTIGEN AND ANTIBODY AT DIFFERENT HYDROGEN ION CONCENTRATIONS.

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(Received for publication, September 21, 1922.)

INTRODUCTION.

In a preceding paper (1) it was observed that the agglutinating power of normal and immune serum increased as the pH approached the acid agglutination optimum of the bacterium under test. Several hypothetical explanations of this phenomenon suggest themselves: (a) The agglutinating power of antibody might be enhanced by incubation at high CH (4.0 to 5.0); (b) the maximal combination of antigen and antibody might take place at this zone; (c) the same amount of antibody in combination might have a greater effect in this range; or (d) the other constituents of the serum may have an agglutinating influence in this range. The following experiments show that the first two suggestions are untenable, and that the third hypothesis accounts for the observations.

Method.

The organism used in these experiments was *Bacillus typhosus*, Pfeiffer strain, obtained through the kindness of Dr. Charles Krumwiede of the Department of Health of the City of New York, to whom we are also indebted for the high titer antityphoid agglutinating serum. The serum dilutions were carried out in glycocoll-Na acetate- Na_2HPO_4 buffer mixtures of definite pH, described in the preceding paper (1). The "standard" bacterial suspensions were prepared as described in the preceding paper (1). Suspensions of higher concentration were used for absorption and other tests, and are always referred to the standard suspensions, as follows: A $10 \times T$

suspension would be one in which the bacteria from 150 cc. of broth were suspended in 15 cc. of distilled water. A $20 \times T$ suspension would be made by suspending the bacteria from 150 cc. of media in 8 cc. of distilled water, and so on.

Experiment 1. Agglutinating Power of Antityphoid Serum Kept at Various C_H for 2 Hours at 43°C.—0.1 cc. quantities of serum > typhoid were diluted with 2.9 cc. of glycoll-acetate-phosphate buffer mixtures (M/12) of various pH from 9.9 to 2.0. Dilution = 1:30. The tubes containing these dilutions were placed in the water bath at 43°C. for 2 hours. 0.1 cc. of each sample was then diluted with 3.35 cc. of buffer mixture pH 6.0. Colorimetric test showed all of the mixtures to be at or very near to pH 6.0. Dilution now approximately 1:1,000. Each cc. contained 0.001 of immune serum. Each sample was now diluted by 2's with buffer solution pH 6.0 to a point where 3.7×10^{-6} cc. of the original immune serum was present in each tube. 1 cc. of standard 4 times washed typhoid suspension was now added to each series of dilutions and incubated at 43°C. for 14 hours. The results are summarized in Table I.

It will be observed from Table I that over a range from pH 9.9 to 3.9, 43°C. for 2 hours has very little effect on the subsequent agglutinating power of antityphoid serum. It is true that a slight decrease in activity manifests itself in Tubes 4 and 5 (pH 6.0 and 5.0). This difference is probably not adventitious, since subsequent tests confirmed the observation. It is probably due to precipitation of the globulin. A really perceptible deleterious effect is observed, however, in Tubes 9 and 10 (pH 2.7 and 2.2). Here a decrease of eightfold loss of agglutinative power is observed in Tube 9, and sixteenfold in Tube 10. The experiment demonstrates that hydrogen ion concentrations of pH 5.0 and 4.6 do not have an enhancing effect on the agglutinating power of the serum, and the first hypothesis has, therefore, to be rejected.

A second explanation of the increased agglutinating activity of serum at pH 4.5 to 5.0 lies in the idea that the combination between antigen and antibody might be more complete in this zone of hydrogen ion concentration. This would be the assumption, *a priori*, of those who believe that the combination of antigen and antibody is influenced by electrical forces (2). Assuming that antibody behaves in a manner similar to serum protein, the former should carry a slight positive charge at pH 4.6. The bacterium, on the other hand, is

negatively charged at this point (1). Therefore, if union between bacterium and antibody is a matter of neutralization of opposite charge, it should be more complete at pH 3.5 to 4.5, where the organism and antibody are oppositely charged, than at pH 6.0 or 7.0, where both bacterium and antibody would be negatively charged.

TABLE I.

Agglutinating Power of Antityphoid Serum Kept at Various C_H for 2 Hours at 43°C.

Tube.	pH at which serum was kept for 2 hrs. at 43°C.	Serum diluted from various pH to pH 6.0; 43°C., 14 hrs., + typhoid suspension.								
		Cc. of serum per cc. of solution.*								
		1×10^{-3}	5×10^{-4}	2.5×10^{-4}	1.2×10^{-4}	6×10^{-5}	3×10^{-5}	1.5×10^{-5}	7×10^{-6}	3.5×10^{-6}
1	9.9	C.	C.	C.	C.	C.	++	+	Tr.	Tr.
2	9.0	C.	C.	C.	C.	C.	++	+	—	—
3	7.5	C.	C.	C.	C.	C.	++	+	Tr.	—
4	6.0	C.	C.	C.	C.	++	+	Tr.	—	—
5	5.0	C.	C.	C.	C.	++	++	+	Tr.	—
6	4.6	C.	C.	C.	C.	C.	++	+	Tr.	—
7	3.9	C.	C.	C.	C.	C.	++	+	+	Tr.
8	3.3	C.	C.	C.	C.	++	++	+	+	Tr.
9	2.7	C.	C.	++	+	—	—	—	—	—
10	2.2	C.	++	+	—	—	—	—	—	—
11	Control serum undiluted; 43°C.; 2 hrs.	C.	C.	C.	C.	C.	++	+	—	—

*Represents concentration of serum (*i.e.* $\frac{1}{\text{dilution}}$ = cubic centimeter of serum per cubic centimeter of solution). Subsequent addition of equal volume of suspension divides this concentration by 2.

The degree of combination is best studied by absorption experiments. Since the absorption of antibody is carried out with concentrated suspensions, it was considered necessary to determine the relationship existing between concentration of the microbic suspension and the amount of serum necessary to cause complete agglutination.

Experiment 2. Relation between Concentration of Bacterial Suspension and Complete Agglutinating Dose of Typhoid Immune Serum.—As described above, the standard suspension used in these typhoid agglutination experiments is pre-

pared by suspending the 4 times washed organisms from 150 cc. of a 16 hour broth culture in 160 cc. of distilled water. Five suspensions were now prepared. Washed organisms from 150 cc. of broth were suspended in 8, 16, 32, 64, and 160 cc. of distilled water, thus securing suspensions of 20, 10, 5, and 2.5 times the concentration of the original. 1 cc. of each of these suspensions was now tested against a series of dilutions of antityphoid serum. Glycocoll-acetate-phosphate buffer mixture, pH 6.3, was used as diluent. Incubation at 43°C. for 14 hours. "Complete agglutination" was considered to take place when the supernatant fluid was water-clear, no whorl of suspended bacteria being visible when the tube was agitated before a strong beam of light. "++" was read when a heavy sediment was present, with a still cloudy supernatant fluid showing a distinct whorl of unagglutinated organisms. The results are summarized in Table II.

TABLE II.

Relation between Concentration of Suspensions and Complete Agglutinating Dose of Antityphoid Serum.

Concentration of T suspension.	Concentration of antityphoid serum $\times 10^{-3} =$					
	150	75	37.5	18	9	4.5
20 times.	C.	++	+	+	Tr.	Tr.
10 "	C.	C.	++	++	++	+
5 "	C.	C.	C.	++	++	+
2.5 "	C.	C.	C.	C.	++	++
1	C.	C.	C.	C.	C.	++

Table II demonstrates that within the limits of suspension concentration studied in this experiment, the amount of serum necessary to agglutinate varies directly with the concentration of the suspension. The results show that at least 16 times less serum is required to agglutinate completely the standard suspension than to cause the same result in the suspension of 20 times the standard. There is reason to believe that the result would have been the same had the suspension varied in exactly the same proportion as the serum dilution, that is to say 16 times. It is therefore justifiable to calculate the agglutinating dose for a suspension 20 times the standard from a result gained from titration of serum on the standard suspension, and *vice versa*.

Experiment 3. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—0.1 cc. quantities of antityphoid serum were diluted 1:30 with glycocoll-acetate-phosphate buffers of pH 7.5, 5.2, 3.9, and 3.3. 3.0 cc. of serum so diluted were then mixed with equal volume of typhoid suspension, 10

times the standard concentration. Parallel control tubes were set up with 3.0 cc., 1:30 serum + 3.0 cc. of distilled water. The eight tubes were incubated at 43°C. for 2 hours, centrifuged at 3,000 R.P.M. for 30 minutes, and the supernatant fluids diluted to 0.001 cc. (1:1,000) with buffer pH 6.0. This brought

TABLE III.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

pH at which absorption took place.	Supernatant fluids after absorption.							Agglutinating doses remaining.*	Agglutinating doses absorbed.
	Concentration in buffer pH 6.0 (cc. $\times 10^{-3}$).								
	100	50	25	12	6	3	1.5		
pH 7.5 Absorbed with $10 \times T$ suspension.	++	-	-	-	-	-	-	<3	>50
pH 7.5 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 5.2 Absorbed with $10 \times T$ suspension.	++	+	-	-	-	-	-	<3	>50
pH 5.2 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 3.9 Absorbed with $10 \times T$ suspension.	C.	C.	C.	++	+	Tr.	-	14	41
pH 3.9 Control.	C.	C.	C.	C.	C.	++	+	55	
pH 3.3 Absorbed with $10 \times T$ suspension.	C.	C.	C.	C.	++	Tr.	-	27	27
pH 3.3 Control.	C.	C.	C.	C.	C.	++	+	55	

* Agglutinating doses calculated for three volumes of 10 times standard suspension, since this was the concentration used in the absorbing suspension.

all supernatant fluids to a common pH. These neutralized diluted supernatant fluids were now diluted with pH 6.0 buffer by 2's to 0.000015 cc. and 1 cc. of standard suspension added to each tube. Incubation for 14 hours at 43°C. The result is given in Table III.

The experiment summarized in Table III shows that at pH 3.9 and more especially at pH 3.3, much less immune body is absorbed than at pH 5.2 and 7.5. There is no sensible difference between the amount absorbed at the latter two hydrogen ion concentrations. In both cases at least 94 per cent of the agglutinating substance was removed. The removal, however, was not complete in any case. Now, the agglutinating power of immune serum is distinctly higher at pH 5.2 than at pH 7.5 (1, 3). Therefore, it is evident that this increased effectiveness cannot be ascribed to a more complete quantitative union at 5.2 than at 7.5.

This experiment was subsequently elaborated and modified in certain respects. In the first place it is desirable, for constant results, to use a more concentrated absorbing suspension. Again the reaction is better conducted at room temperature than at 43°C., since at lower pH (3.3 to 3.0) the destructive effect of temperature on immune body begins to be a marked one. Finally, it seemed wise to conduct the experiment at a greater number of CH , thus filling in the gaps existing in Experiment 3.

Experiment 4. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—2.0 cc. quantities of antityphoid horse serum, diluted 1:50 in buffer mixtures, pH 7.5, 5.2, 4.7, 3.9, 3.3, 3.0, and 2.7 were mixed with 2.0 cc. amounts of *B. typhosus* suspension 20 times the concentration of the standard (organisms from 150 cc. broth suspended in 8 cc. distilled water). Similar amounts of immune serum diluted with buffers of same pH were mixed with equal amounts of distilled water. These latter served as controls. All tubes were allowed to stand at room temperature (18°C.) for 11 hours.

The tubes with the immune serum *B. typhosus* mixtures were then centrifuged at 3,000 R.P.M. for 30 minutes. The supernatant fluids, together with the controls (immune serum + distilled water) were now diluted 10 times with buffer pH 6.5, bringing all mixtures to that pH.

These dilutions, equivalent to 0.001 cc. of original serum, were diluted by 2's to 0.000007 cc. of the original; 1 cc. of standard 4 times washed distilled water suspension was added to each tube, and all were incubated at 43°C. for 14 hours.

The smallest amount causing complete agglutination was taken as the criterion for the agglutinating unit. The agglutinating units recorded after the last incubation (supernatant fluids + standard suspension) were calculated to the agglutinating units required for the absorbing suspension (20 times the standard). This was done by dividing by 40 (since twice the volume of suspension was used in the adsorption experiment), in accordance with Experiment 2.

The results are given in Table IV.

TABLE IV.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

pH	Tube.	Mixture.	Total agglutinating units present.*	Units remaining after 11 hrs. at 18° C.	Units absorbed.
7.5	1	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	2	Antityphoid serum + distilled water. Control.	16	16.0	0
5.2	3	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	4	Antityphoid serum + distilled water. Control.	16	16.0	0
4.7	5	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	6	Antityphoid serum + distilled water. Control.	16	16.0	0
3.9	7	Antityphoid serum absorbed with concentrated suspension.	16	1.3	14.7
	8	Antityphoid serum + distilled water. Control.	16	16.0	0
3.3	9	Antityphoid serum absorbed with concentrated suspension.	16	2.0	14.0
	10	Antityphoid serum + distilled water. Control.	16	16.0	0
3.0	11	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	12	Antityphoid serum + distilled water. Control.	16	16.0	0
2.7	13	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	14	Antityphoid serum + distilled water. Control.	16	16.0	0

* Calculated for 2 cc. of 20 times the standard suspension.

Control experiments on the effect of time showed that the combination was almost instantaneous, in agreement with results on the potential (1). The results therefore are not due merely to a different speed of combination but to a difference in the final stage.

Table IV confirms the conclusion drawn from Experiment 3; *i.e.*, that there is no demonstrable difference between the amount of immune body absorbed at pH 4.7 and that taken up at 5.2 and 7.5.

Relation of the Results of the Absorption and Agglutination Experiments.

The experiments described in the preceding paper (1) show that the amount of immune serum or of protein required to agglutinate bacteria depends entirely on the pH at which the experiment is performed. The nearer the pH is to the acid agglutination zone of the organism in question, the smaller the amount of serum required to agglutinate. This result has also been obtained by Michaelis and Davidsohn (3) and by Eggerth and Bellows (4). The present experiments show that this result cannot be ascribed to an increase in the amount of antibody combined in this region nor to any increase in the amount of immune body. These results also show that the combination between organism and immune body is independent of the charge, since the amount of immune body combined at pH 4 to 5, where the organism is negative and the immune body positive, is the same as in the range between 5 and 8, where both are negative. The influence of salt on the combination of antibody and organism also contradicts the idea that the combination is caused by opposite electric charges. If this were the case the combination should be greatest when the charges were greatest. Salt, however, decreases the charge but increases the combination. The same fact is brought out by Loeb's (7) experiments with collodion treated with gelatin. The collodion is always negative yet will become coated with alkaline gelatin solutions in which the gelatin is also negative.

The present experiments could be explained by assuming that the immune body was always *positive* and that the reversal found by Michaelis (and which was confirmed in the course of this work) was due to the fact that the immune body was carried along with the globulins as is the case with pepsin (Pekelharing and Ringer (5)). This explanation, however, could not account for the experiment shown in Fig. 3 of the preceding paper (1), which shows that at a pH of 3.0, where the organisms themselves are positive, the addition of serum *increases* this positive charge. It would seem to be improbable

that an increase in a positive charge could be caused by combination with a substance having a negative charge.

The experiments may be simply accounted for if it be supposed that the antibody or protein forms a film on the surface of the organism and is held by the ordinary forces of valence. This mechanism has been found by Langmuir (6) to exist in the case of oil films on water. The portion of the surface that is covered would then acquire the characteristics of the added substance. It is evident from this point of view that the smaller the charge on the original particle, the smaller the amount of the surface which would have to be covered in order for the particle to have the charge of the added substance; in other words, the less the amount of substance necessary to agglutinate. This is the experimental fact. This is clearly shown in Fig. 1 in which the charge on the organisms, the amount of serum to agglutinate, and the amount of antibody combined, are plotted against the pH. The figure shows that there is no relation between the amount of antibody in combination and the amount required to agglutinate, but a very close connection between the amount required to agglutinate and the charge carried by the organism.

The independence of the agglutination and sign of charge is also shown in Table V. In this experiment the amount of serum necessary to agglutinate organisms in NaCl in which they are negative is compared to the amount of serum required to agglutinate the same suspension in Cu acetate in which they carry an equal positive charge.

The mechanism of film formation will also account for the results of Arkwright (8) who found that *Bacterium coli*, sensitized with washings from *Bacillus typhosus*, could be agglutinated by anti-*Bacillus typhosus* serum. It was found that the same experiment could be performed with egg albumin. *Bacillus typhosus*, in the presence of egg albumin, could be agglutinated with anti-egg albumin serum.

These experiments show, therefore, that the combination between organism and agglutinin (or protein) is not *due to* opposite electric charges, but that the effect on the charge is the *result* of the combination.

TABLE V.

Precipitation with Immune Serum in the Presence of Copper Acetate or Sodium Chloride.

20 cc. one-half suspension + 40 cc. copper acetate or sodium chloride of concentration noted.

2 cc. resulting suspension + 0.5 cc. antityphosus rabbit serum.

Final concentration of salt.	Copper acetate 0.10 N.	Sodium chloride 0.02 N.
Migration velocity under potential gradient of 1 volt per cm., μ per sec.	+0.50	-0.56
Potential millivolts.	+6.5	-7.0
Concentration of immune serum.	Agglutination after 20 hrs.	
1:4 } Immediate precipitation of copper	C.	C.
1:12 } hydroxide and serum protein.	C.	C.
1:36 }	C.	C.
1:108	C.	C.
1:324	C.	C.
1:976	+++	C.
1:2,700	+++	C.
1:8,100	++	+++
1:2,500	++	+++
1:7,500	++	+
1:22,000	+	+
0	$\pm$	-

SUMMARY.

1. The amount of immune body required to agglutinate a suspension of *Bacillus typhosus* increases in direct proportion to the concentration of the suspension.

2. The amount of immune body combined with the organisms is constant from pH 9 to pH 3.7. Below the latter value the amount in combination is decreased.

3. The addition of immune serum to a suspension of *Bacillus typhosus* at a pH of 2.5 increases the positive charge of the organisms.

These results are contradictory to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and the organism. They agree with the assumption that the immune body forms a film on the surface of the organism and that the effect on the charge is the result of this film.

BIBLIOGRAPHY.

1. Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 639, 655.
2. This point of view is clearly presented by Landsteiner, K., in Kohle, W., and von Wassermann, A., *Handbuch der pathogenen Microorganismen*, Jena, 2nd edition, 1913, ii, 1241.
3. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.
4. Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921-22, iv, 669.
5. Pekelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.
6. Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.
7. Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922.
8. Arkwright, J. A., *J. Hygiene*, 1914, xiv, 261.

THE STABILITY OF BACTERIAL SUSPENSIONS.

V. THE REMOVAL OF ANTIBODY FROM SENSITIZED ORGANISMS.

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(Received for publication, September 21, 1922.)

The experiments described in the preceding paper (1) show that the combination of antibody and organism in the case of *Bacillus typhosus* is less on the acid side of pH 4.0 than on the alkaline side. It seemed possible, therefore, that antibody could be more completely removed by washing at pH 3.0. Experiment showed, however, that this was not the case. It was further found that much more antibody could be recovered by washing with distilled water than by washing with salt solution. This is in confirmation of the results of Huntoon (2) and other workers. The result of such an experiment is given in Table I. The technique was the same as that described in the preceding paper. It is evident that some of the immune body is easily removed but that the equivalent of about twelve agglutinating doses remains on the organisms. The suspension which had originally less than this number did not give up any measurable immune body. If it be supposed that the immune body forms a film on the surface of the organism, this is the result that would be expected. It has been shown by Langmuir (3) in the case of gaseous films that the first layer of molecules can be removed only with the greatest difficulty, whereas the succeeding ones are relatively easily removed. It was shown in a former paper that with optimum salt concentration agglutination occurred with one-eighth to one-sixteenth of the amount of serum required to cause agglutination in the most dilute salt. This result agrees with the present experiment in indicating that agglutination will occur when the surface is about one-eighth covered and therefore that a complete surface film represents about eight agglutinating doses.

TABLE I.

Recovery of Immune Body from Bacterial Sediments.

Supernatant fluid.	pH at which sediment had absorbed immune body.	Supernatant fluid diluted with buffer pH 6.3, 43°C., 14 hrs.						Units in sediment before washing.	Recovered.	Anti-body remaining on organism.
		0.5	0.25	0.125	0.06	0.03	0.015			
Supernatant fluid No. 1 from buffer pH 7.5 treatment.	7.5	++	Tr.	—	—	—	—	16	—	16
	5.2	+	—	—	—	—	—	16	—	16
	4.7	+	Tr.	—	—	—	—	16	—	16
	3.9	+	Tr.	—	—	—	—	14.7	—	15
	3.3	++	Tr.	—	—	—	—	14	—	14
	3.0	++	Tr.	—	—	—	—	7.8	—	7.8
Supernatant fluid No. 2 from distilled water treatment.	7.5	C.	C.	C.	C.	++	Tr.	16	4.1	12
	5.2	C.	C.	C.	C.	++	+	16	4.1	12
	4.7	C.	C.	C.	C.	++	Tr.	16	4.1	12
	3.9	C.	C.	C.	++	++	Tr.	16	4.1	12
	3.3	C.	C.	++	+	—	—	14	1.7	12.3
	3.0	Tr.	—	—	—	—	—	8.2	<1	7.8

TABLE II.

Effect of Repeated Distilled Water Washing on the Acid Agglutination Optimum of B. typhosus Laden with Immune Body.

Series.	No. of washings of sediment + immune body	43°C. 14 hrs								3.3 = pH of buffer.
		7.9	7.5	6.6	6.0	5.5	5.0	4.4	4.0	
1	Not washed.	++	++	C.	C.	C.	C.	C.	C.	C.
2	1	+	+	+	++	++	C.	C.	C.	C.
3	2	Tr.	+	+	++	++	C.	C.	C.	C.
4	3	Tr.	Tr.	Tr.	+	+	++	C.	C.	C.
5	4	—	Tr.	Tr.	+	+	++	C.	C.	C.
6	5	—	Tr.	Tr.	+	+	++	C.	C.	C.
7	6	—	—	Tr.	+	+	++	C.	C.	C.
8	7	—	—	Tr.	Tr.	+	+	C.	C.	C.
9	8*	—	—	Tr.	Tr.	Tr.	+	++	C.	C.
10	Control suspension (no immune body).	—	—	Tr.	Tr.	Tr.	Tr.	+	C.	C.

* Stood over night at room temperature in distilled water, 3.0 cc.

The question arises whether all the immune body can be removed if washing is sufficiently prolonged. This cannot be determined by testing for the recovered antibody, since the solution is so dilute that no reaction is obtained.

It was shown in a preceding paper (4) that the immune serum widened the acid agglutination zone and moved it to the alkaline side. The limit of acid agglutination, therefore, serves as a sensitive test for the presence of immune body. The following experiment shows that sufficient washing removes all the immune body.

6 cc. serum anti-*typhosus* (1:50 in buffer pH 6.3) + 6 cc. of 10 times standard *typhosus* suspension, 18°C., 5 minutes.

The mixture was centrifuged, the supernatant fluid pipetted off, and the sediment resuspended in 10 cc. of distilled water.

1 cc. of the resulting heavy suspension was diluted to 10 cc. with distilled water, and of this second suspension 1 cc. amounts were tested against buffer series pH 7.9 to 3.3.

The balance of the heavy suspension (9 cc.) was centrifuged, resuspended in 9 cc. of distilled water, 1 cc. removed, diluted to 9.0 in distilled water, as before, and the resulting suspension tested against the same buffer series.

This process was repeated 8 times, the distilled water for washing being reduced in amount by 1 cc. each time. This was done to keep the concentration of the original suspension of organisms constant, since 1 cc. was removed at every washing for the preparation of the dilute suspension to be tested against the buffer series.

Table II shows that the acid agglutination zone of organisms laden with immune body is greatly broadened. Thus in Series 1, Table II, the unwashed sediment is agglutinated completely at pH 6.6, and ++ agglutination occurs at pH 7.5 and 7.9. As washing proceeds, and the immune body is removed, progressively higher C_H is necessary to cause complete flocculation. At the eighth washing (Series 9) the agglutinability by acid has returned practically to that of organisms to which no immune body has been added—Series 10. The immune body can therefore be completely removed by sufficient washing.

This experiment suggests the use of this method as a test for the presence of agglutinins in bacterial suspensions. A modification of the method would be to determine the salt concentration required for agglutination, since this is also a sensitive test for immune body.

SUMMARY.

1. The removal of antibody from *Bacillus typhosus* is no more complete at pH 3 than at pH 7.

2. Approximately twelve agglutinating doses are firmly combined with the organisms. Immune body in excess of this amount is easily removable by distilled water.

3. A method of testing for the presence of immune body on the organism is described which depends on the difference in the acid agglutination of sensitized and unsensitized organisms.

4. Repeated washing in distilled water will serve to remove all the immune body from sensitized bacteria.

BIBLIOGRAPHY.

1. De Kruif, P. H., and Northrop, J. H., *J. Gen. Physiol.*, 1922-23, v, 127.
2. Huntoon, F. M., *J. Immunol.*, 1921, vi, 117.
3. Langmuir, I., *J. Am. Chem. Soc.*, 1916, xxxviii, 1145.
4. Northrop, J. H., and DeKruif, P. H., *J. Gen. Physiol.*, 1921-22, iv, 655.

SODIUM CHLORIDE AND SELECTIVE DIFFUSION IN LIVING ORGANISMS.

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(Received for publication, September 5, 1922.)

I.

Proof that NaCl Acts Similarly to CaCl₂ and LaCl₃ on the Rate of Diffusion of Acid into the Egg of Fundulus.

1. Each organism and each cell is in dynamic equilibrium with its surroundings, which means that the chemical changes which occur in the cells are, within certain limits, automatically reversed. Although an understanding of the mechanism of this regulation is one of the fundamental problems of physiology, large gaps exist in our knowledge of these regulatory processes. Why is it, for instance, that the blood of bony fishes can maintain a concentration in NaCl far below that of the ocean and far above that of the fresh water in which they may be living? Why is it that the red blood corpuscles¹ or the cells of muscles (and probably cells in general) remain rich in potassium and entirely or almost free from sodium, while the blood is rich in sodium and poor in potassium? Why is it that the waste products, which are formed in one part of the body, diffuse into the blood but do not, as a rule, diffuse into the other cells of the body?

It is the intention of this paper to show that the salts which surround a cell modify the properties of the cell wall in such a way as to accelerate the rate of diffusion of certain ions and retard the rate of diffusion of other ions, and that aside from calcium chloride, sodium chloride is a very important factor in causing such selective diffusion.

Over 20 years ago the writer made the observation that the newly fertilized eggs of the marine fish *Fundulus* which normally develop in sea water, are killed when put into a pure NaCl solution of that

¹ Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1922, liii, 241.

concentration in which NaCl exists in the sea water, while they can live indefinitely in such a solution when a small but definite quantity of a salt with bivalent cation (especially CaCl_2) or a still smaller concentration of a salt with a trivalent cation is added.² When the eggs are older, they can live for a number of days in a 2.5 M solution of NaCl when the proper amount of CaCl_2 is added, while in a pure NaCl solution of that concentration they are killed in a few hours. Since these eggs can develop as well in distilled water as in sea water, it was obvious that the Ca or the other plurivalent cations were not required for any nutritive purpose. In 1905 the writer suggested as an explanation that a pure NaCl solution, if its concentration exceeded a certain limit, made the membrane of the egg more permeable, so that NaCl could diffuse into the egg, killing the embryo; while this increase in permeability was prevented by the presence of a low concentration of Ca.³ The writer's further experiments on *Fundulus*⁴ left little doubt concerning the correctness of this suggestion and it was proven beyond doubt and shown to be a general phenomenon by the beautiful experiments of Osterhout⁵ and his collaborators on *Laminaria* and *Nitella*.

While these experiments were correct as far as the rôle of Ca (and other bivalent cations) was concerned, they were incomplete. In these experiments CaCl_2 appeared as an antagonist to NaCl, which it was under the conditions of the experiments in which an excess of NaCl was used. The experiments, however, were incomplete inasmuch as they failed to show another equally important fact, namely, that in moderate concentrations NaCl acts exactly like CaCl_2 or LaCl_3 , and that sodium chloride is, perhaps, as important in determining selective diffusion of ions through the cell wall as is CaCl_2 .

In 1910 Wasteneys and Loeb observed that the marine fish *Fundulus*, which can also live in distilled water, died in 3/10,000 M HNO_3 in less than an hour, while the fish lived indefinitely when the same

² Loeb, J., *Am. J. Physiol.*, 1899-1900, iii, 327; 1901-02, vi, 411. *Arch. ges. Physiol.*, 1901-02, lxxviii, 68.

³ Loeb, J., *Arch. ges. Physiol.*, 1905, cvii, 252.

⁴ Loeb, J., *Science*, 1912, xxxvi, 637; 1916, xlv, 574; *Biochem. Z.*, 1912, xlvii, 127.

⁵ Osterhout, W. J. V., *Injury, recovery, and death, in relation to conductivity and permeability*, Philadelphia and London, 1922.

HNO₃ solution was made up in N/10 NaCl. CaCl₂ was even more effective as an antagonist to acid than NaCl.⁶ Later the inhibiting effect of salt on the toxic action of acids was observed in the egg of the same fish.⁷

We will show in this paper that NaCl, CaCl₂, and LaCl₃ act all alike, each one retarding the rate of diffusion of acid through the membrane of *Fundulus* and thereby preventing or retarding the toxic action of the acid on the embryo, with this difference only—that the minimal concentration of the salt in which this inhibiting effect occurs diminishes rapidly with the valency of the cation of the salt.

Two different sets of experiments were made. In one set a certain number of eggs were put into a definite quantity of an acid solution of a definite hydrogen ion concentration and the rate at which acid diffused into the egg was determined by measuring the pH of the solution colorimetrically at different intervals. Since the quantity of acid present in the solution was known in the beginning, it was possible to calculate from the change in pH how much acid had diffused into the egg. In this way the influence of salts on the rate of diffusion of acid into the egg could be measured by following the change of pH of the solution colorimetrically.

In the second set of experiments, twenty eggs were always put into 50 cc. of an acid solution of a definite pH with or without salts, and after a certain number of hours, the percentage of eggs in which the hearts were no longer beating was determined. It may be assumed that the heart of the embryo stops beating when a certain quantity of acid has diffused into the egg, and that this quantity is approximately the same for each heart at the same age of the embryo. There are, however, individual variations in the membranes as a consequence of which the rate of diffusion of acid is not the same for different eggs. Assuming that these individual variations are distributed equally in each group consisting of a larger number of eggs, we may further assume that the percentage of hearts which have stopped beating in each group after a certain time is the expression of the average quantity of diffusion of acid during this time. In this way we gain

⁶ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

⁷ Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii, 147.

also a measure of the influence of salts on the rate of diffusion of acid through the membrane with the aid of mortality curves.

The eggs used were about 5 days old or older, the heart beat and circulation of the embryos being well developed. The eggs were kept for at least 2 days previous to the experiment in distilled water to free the outside of the membrane from salts. This is absolutely necessary for the study of the influence of salts on the rate of diffusion of acid into the eggs. Twenty eggs which had previously been examined to make sure that their hearts were beating normally were put into an Erlenmeyer flask containing 50 cc. of an acid solution, generally (unless the contrary is stated) HCl of pH 3.7. These acid solutions were made up in either H_2O or salt solution and they were brought to the desired pH with the aid of the proper indicators. It was necessary to compare only such experiments with each other as were made simultaneously to eliminate the influence of temperature. After a certain time the eggs were examined microscopically to determine the percentage of eggs in which the hearts had stopped beating. The pH of the supernatant acid solution was then determined colorimetrically to check the results. It may be said that the colorimetric check was always parallel to the percentage of hearts which had stopped beating. We will first give the results of the influence of salts on the rate of diffusion of acid into the egg by the method of mortality curves.

2. In Fig. 1 are found the mortality curves for HCl of pH 3.7 in the presence of different concentrations of NaCl, $CaCl_2$, and $LaCl_3$. The abscissæ are the concentrations of the salts, the ordinates the percentage of eggs killed by the acid (the stopping of the heart beat by acid becomes rapidly irreversible after a certain time). When all were killed, the value of the ordinate in these mortality curves is 100. The duration of the experiment given in Fig. 1 was about 17 hours. In 17 hours 90 per cent of the eggs were killed by the acid when no salt was present; when salt was present the mortality was less, but the retarding influence of the three different chlorides NaCl, $CaCl_2$, and $LaCl_3$ on the diffusion of acid into the egg increased rapidly with the increasing valency of the cation. Thus to reduce the mortality of eggs in 50 cc. of HCl of pH 3.7 from 90 per cent to 40 per cent, $m/500,000$ $LaCl_3$ (or $CeCl_3$ or $AlCl_3$) was sufficient, while a con-

centration of between $m/8,192$ and $m/4,096$ $CaCl_2$, and of $m/64$ $NaCl$ was required. In order to overcome completely the toxic effect of HCl of pH 3.7 (*i.e.* to prevent completely the diffusion of acid into the egg) about $m/32,000$ to $m/16,000$ $LaCl_3$ (or $CeCl_3$ or $AlCl_3$), about $m/1,024$ to $m/512$ $CaCl_2$, and, as we shall see presently, about $m/8$ to $m/4$ $NaCl$ were required. The influence of the valency of the

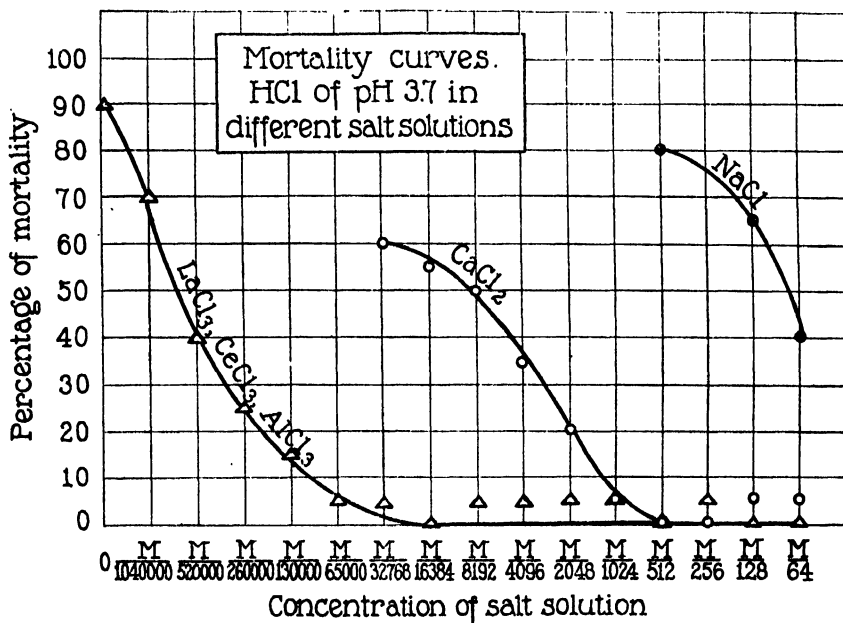


FIG. 1. Inhibiting influence of salts on the toxic effects of HCl solution of pH 3.7. Abscissæ are the concentrations of salts; ordinates, percentage of eggs which died in 18 hours. $LaCl_3$, $CeCl_3$, $AlCl_3$, $CaCl_2$, and $NaCl$ inhibit the toxic effects of acid, but the concentration of salt required diminishes rapidly with the valency of the cation.

cation on the rate of diffusion of HCl of pH 3.7 into the egg is therefore enormous, Ca being about 100 times as effective as Na , and La being at least 40 times as effective as Ca . What interests us here is the fact that $NaCl$ acts qualitatively like $CaCl_2$ and $LaCl_3$, and that only the concentration of salt required differs. The concentration of maximal efficiency of $NaCl$, namely $m/8$ to $m/4$, is that in which it exists in the blood of mammals and many cold blooded vertebrates.

This striking valency effect of the cations suggested a number of additional experiments; namely, first, a comparison of the effect of Na_2SO_4 with that of NaCl . In this case the experiments lasted 3 days. The results are found in Fig. 2. It is obvious that the inhibiting action of Na_2SO_4 on the diffusion of acid is greater than that of NaCl and that therefore the SO_4 ion cannot be said to have an

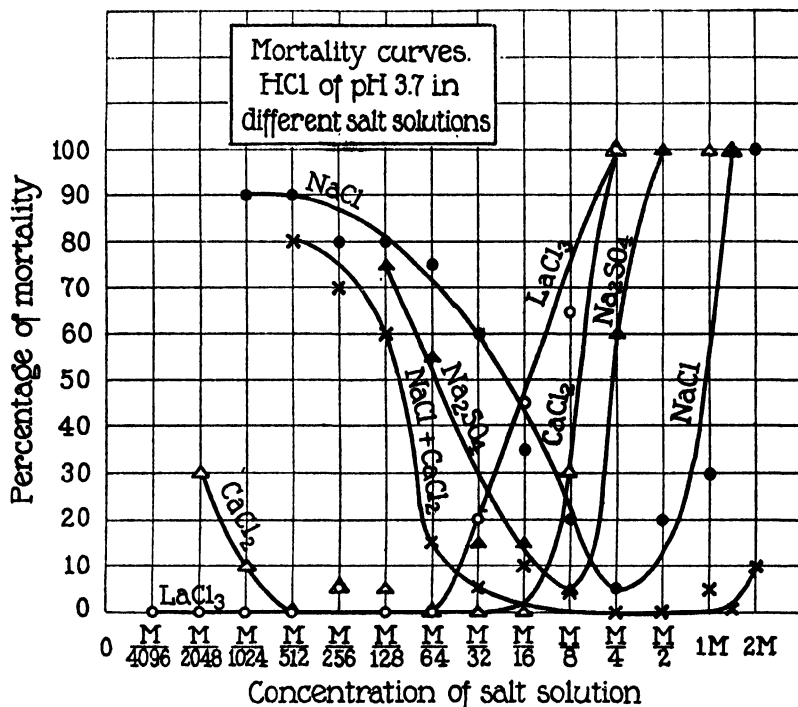


FIG. 2. The inhibiting effect of Na_2SO_4 is greater than that of NaCl but less than that of a balanced solution of $\text{NaCl} + \text{CaCl}_2$. At higher concentrations this inhibiting effect of salt on the toxic action of acid disappears again.

effect opposite to that of the cation. Fig. 2 gives also a comparison of the inhibiting effect of NaCl alone and of $\text{NaCl} + \text{CaCl}_2$ (in the proportion of 100 molecules of NaCl to 1.5 molecules of CaCl_2). It is obvious that the inhibitory effect of $\text{NaCl} + \text{CaCl}_2$ on acid is greater than that of NaCl alone, and the effects of NaCl and of CaCl_2 seem to be additive. Complete inhibition of the toxic effect of acid (or rather complete inhibition of the diffusion of acid into the egg)

during the 3 days was caused by LaCl_3 at a concentration below $\text{m}/4,096$, by CaCl_2 at a concentration between $\text{m}/256$ and $\text{m}/16$, by NaCl at $\text{m}/4$, by Na_2SO_4 at $\text{m}/8$, and by a mixture of $\text{NaCl} + \text{CaCl}_2$ (balanced solution) at $\text{m}/16$.

When the concentration of salts exceeds the concentrations mentioned, they cease to inhibit the diffusion of acid into the egg (Fig. 2). LaCl_3 becomes entirely ineffective at a concentration of $\text{m}/8$, NaCl at a concentration of 1 m , CaCl_2 at a concentration of $\text{m}/4$, Na_2SO_4 at $\text{m}/4$, while the balanced solution $\text{NaCl} + \text{CaCl}_2$ remains efficient even at a concentration of 2 m . It is impossible to connect the concentrations at which the salts no longer prevent the diffusion of acid with any purely electrostatic action of ions, and the increase in permeability caused by higher concentrations of salts must be ascribed to an alteration of the membrane due to other than mere electrostatic effects.

This conclusion is corroborated by experiments on the influence of electrolytes other than those thus far mentioned on the rate of diffusion of acid into the egg (Fig. 3). It is obvious that according to the mortality curves in Fig. 3 the inhibiting effect of MgCl_2 on the diffusion of acid is very much less than that of CaCl_2 and almost approaches that of LiCl or NaCl ; and the inhibiting effect of BaCl_2 is only slightly greater than that of MgCl_2 . On the other hand, Mn acts almost as well as Ca . Sr also acts almost like Ca .

These latter facts are, however, only of secondary importance for our purpose which is to show that in the proper concentration NaCl acts exactly like CaCl_2 and like LaCl_3 on the rate of diffusion of acid through the membrane of the *Fundulus* egg (and, perhaps, through the membranes of cells in general).

3. We will now give the results of the second method of following the diffusion of acid into the eggs colorimetrically. HCl solutions were made up in water or salt solutions to a pH 3.8, and ten eggs, about 5 or 6 days old (with beating hearts) were put into 10 cc. of these HCl solutions of pH 3.8 in a test-tube. 1 drop of methyl orange was added to each solution and the test-tubes were closed with rubber stoppers. From time to time (about every 10 minutes) the test-tubes were slightly agitated to change the solution in immediate contact with the eggs. The color of the indicator made it possible to follow the rate of diffusion of the acid into the egg. It was found

that when salt was present in proper concentration, the color of the solution changed very little or not at all during 20 hours at about 22°C., while when no salt was present the color changed, betraying a gradual diminution of the acid in the solution. By comparing the color of the solution with standardized indicator solutions, the change in pH could be ascertained. Fig. 4 contains some of the results. The ordinates are the pH of the acid solutions while the abscissæ are the times.

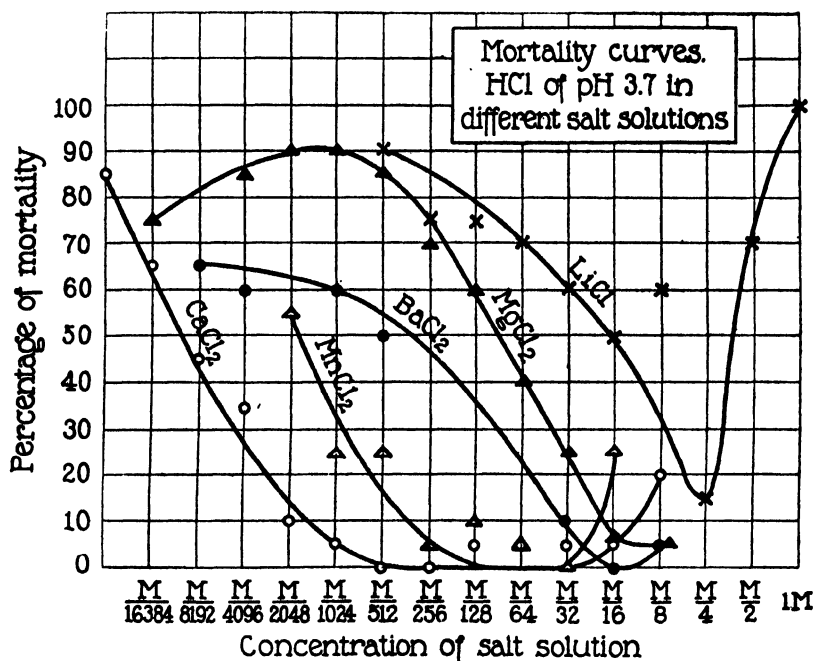


FIG. 3. Proof that the inhibiting effect of $MgCl_2$ and $BaCl_2$ on the rate of diffusion of acid into the egg is less than that of $CaCl_2$.

It is seen from Curve I in Fig. 4 that without salt the pH of the solution rose gradually in 7 hours from 3.8 to pH 4.2. Since there were 10 cc. of solution of HCl present the amount of acid which diffused into the ten eggs can be calculated from the curve. We may omit the calculation, since it is of no special interest in this connection. After 20 hours the pH was over 5.0; *i.e.*, practically all the acid had diffused from the solution into the eggs, and all the embryos were dead

after 20 hours. Curve II in Fig. 4 shows that in $M/8$, $M/4$, and $M/2$ NaCl the pH of the solution did not change during the 20 hours of the experiment; *i.e.*, practically no acid diffused into the eggs and the embryos were all alive after that time. Curve III shows that in

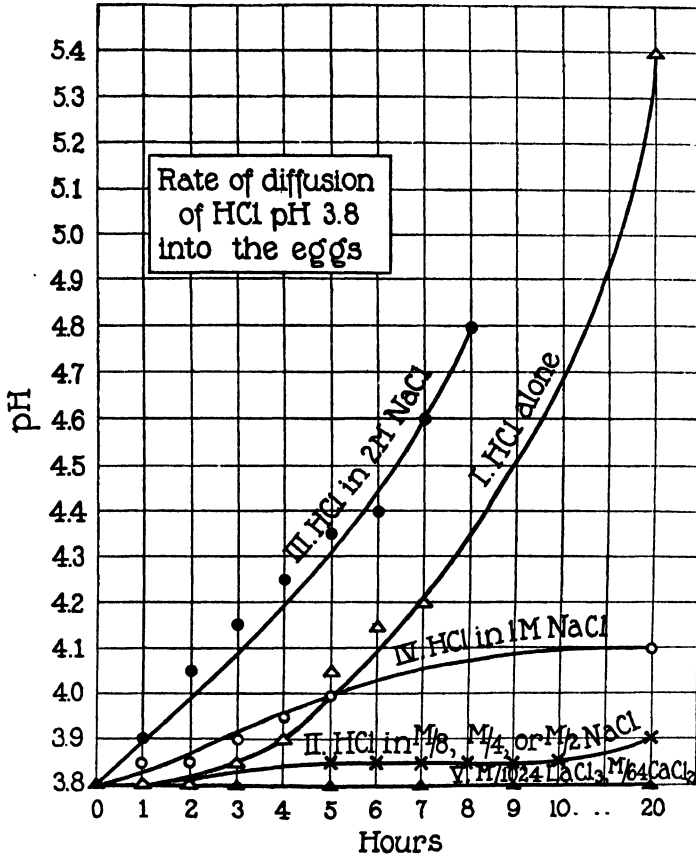


FIG. 4. Influence of salts on rate at which acid diffuses into the egg. Abscissæ are the time in hours, ordinates the pH of the acid solution. The original pH of the acid solution was 3.8.

2 M NaCl the acid diffused even more rapidly into the eggs than without salt and all the embryos were killed rapidly. In 1 M NaCl (Curve IV) the acid diffused first rapidly and later more slowly than without salt; seven of the ten eggs were killed after 20 hours. In $M/1,024$ $LaCl_3$ and $M/64$ $CaCl_2$ (Curve V) no color change occurred in the solution

and no acid diffused into the eggs in 24 hours or more and all the embryos remained alive.

In all experiments with mortality curves the pH of the supernatant solution was measured at the end of the experiments. Thus Fig. 5 shows that the curves for the change in pH for NaCl and NaCl +

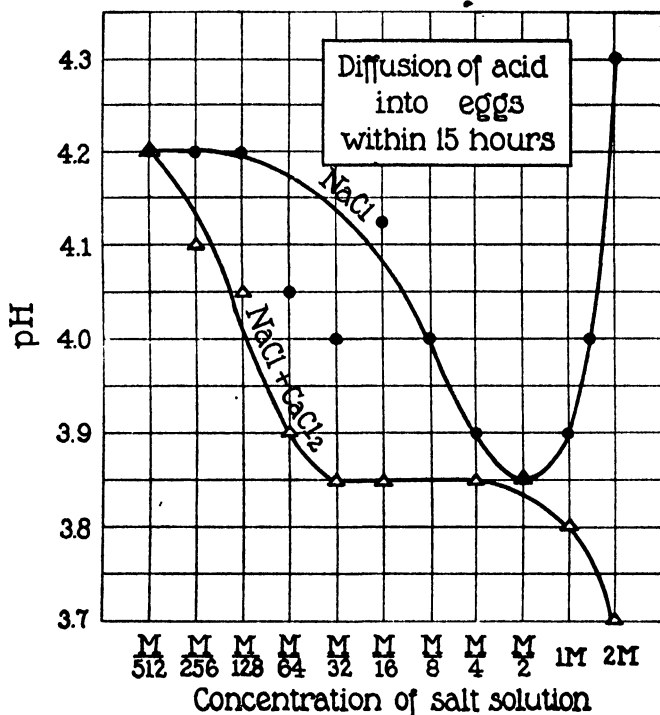


FIG. 5. Change of pH of HCl solution after 15 hours. Original pH 3.7. Abscissæ are the concentrations of NaCl and NaCl + CaCl₂; ordinates the pH of the HCl solution after 15 hours. The two curves are similar to the two mortality curves for the same two solutions in Fig. 2.

CaCl₂ respectively at the end of the experiment corresponded to the mortality curves for these two solutions in Fig. 2. Where the mortality was lower, the pH curve was also lower, and since the mortality was lower in NaCl + CaCl₂ than in NaCl (Fig. 2), we had a right to expect that the same would be true for the pH curves of the two mixtures, which was the case as Fig. 5 shows.

We therefore reach the conclusion that NaCl acts like CaCl_2 or LaCl_3 in preventing the diffusion of strong acids through the membrane of the egg of *Fundulus*. The only difference is that the concentration in which this salt effect is complete is considerably higher in the case of NaCl than in the case of CaCl_2 or LaCl_3 . The greatest efficiency of NaCl is between $m/8$ and $m/2$; i.e., that concentration in which NaCl exists in the sea water and the blood of the majority of animals.

II.

Proof of the Lack of Inhibiting Action of Salts on the Diffusion of Non-Dissociated Weak Acids.

According to Hantzsch, fatty acids, like acetic acid, occur in two tautomeric forms, true acids (which always undergo complete electrolytic dissociation), and pseudo-acids which are not dissociable. There exists an equilibrium between the two forms which upon the addition of a salt like Na acetate shifts in favor of the non-dissociable pseudo-acid form.

As long as the concentration of acid is not too high, so that there is not too high a concentration of pseudo-acid, acetic acid acts much like HCl in the egg of *Fundulus* and the rate of diffusion of acetic acid into the egg of *Fundulus* is retarded by the presence of salt. If, however, much pseudo-acid is present, the egg is no longer protected by salts.

Fig. 6 shows the mortality curve of *Fundulus* eggs in acetic acid, HCl, H_2SO_4 , and H_3PO_4 , plotted over the pH as abscissæ with the percentage of mortality (in 14 hours) as ordinates. It is obvious that acetic acid of the same pH is a little more toxic than HCl and this difference may be ascribed to undissociated acid. The fact that fatty acids are more efficient physiologically than HCl of the same hydrogen ion concentration has been known for a long time. Richards⁸ observed it as far as the minimal concentration of acid for sour taste is concerned, the writer observed it in regard to the effect of the velocity of swelling of muscle in acid,⁹ and later in a most striking

⁸ Richards, T. W., *Am. Chem. J.*, 1898, xx, 121.

⁹ Loeb, J., *Arch. ges. Physiol.*, 1898, lxxi, 457.

way in his experiments on membrane formation in sea urchin eggs in which CO_2 and fatty acids were very efficient and strong mineral acids inefficient.¹⁰ He explained the phenomenon on the assumption of the greater diffusibility of the non-dissociated acid molecule.¹⁰ As soon as the non-dissociated acid molecule gets through the cell wall, it will be transformed from the non-dissociable pseudo-acid form into the dissociable-acid form, producing the acid effect, e.g., sour taste, swelling of muscle (due to formation of a dissociable pro-

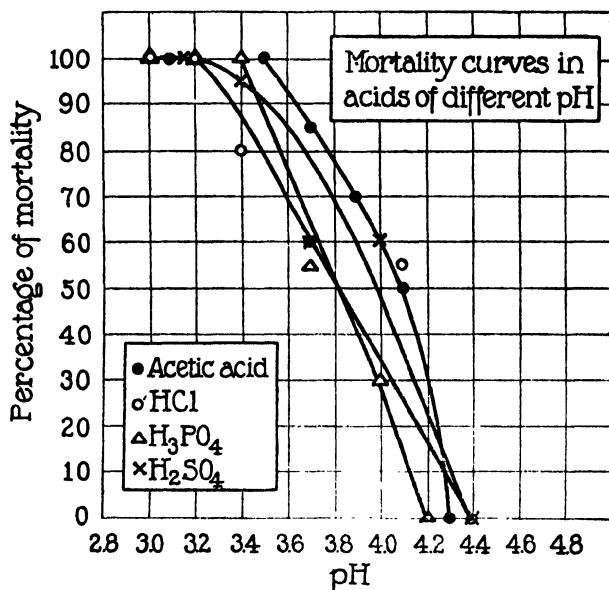


FIG. 6. Mortality curves for different acids. Acetic acid is more toxic than HCl of the same pH, especially at lower pH where the quantity of undissociated acetic acid becomes greater.

tein-acid salt and the establishment of a Donnan equilibrium), or membrane formation in the case of the sea urchin egg. Jacobs more recently demonstrated beautifully the greater diffusibility of non-dissociated acid through living membranes.¹¹

It is possible to retard the diffusion of HCl and of acetic acid of pH 3.8 by NaCl, but this inhibitory effect of NaCl is much less in the

¹⁰ Loeb, J., *Artificial parthenogenesis and fertilization*, Chicago, 1913.

¹¹ Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

case of the diffusion of acetic acid than in the case of HCl. This is shown by the mortality curves in Fig. 7. Thus when HCl of pH 3.8 was dissolved in $M/32$ NaCl, only 20 per cent of the embryos had been killed after 12 hours, while when acetic acid of pH 3.8 was dissolved in $M/32$ NaCl, 40 per cent were killed. To keep all the eggs alive for 12 hours in acid of pH 3.8, $M/16$ NaCl was required in the case

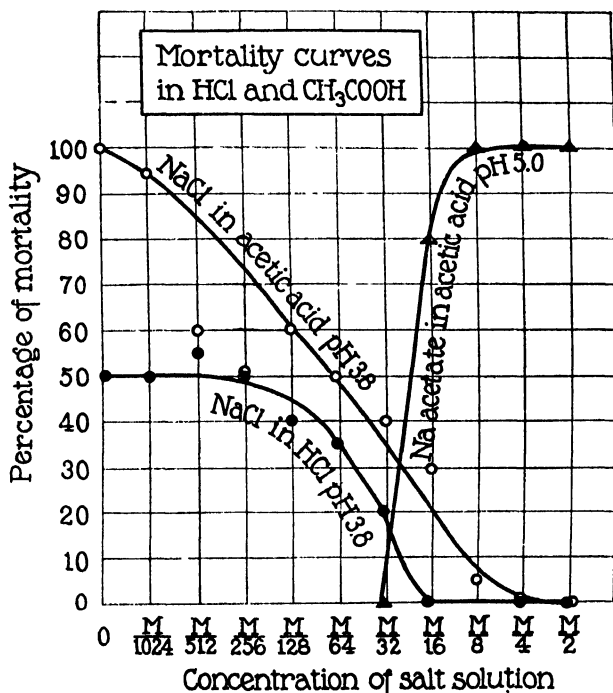


FIG. 7. Showing that NaCl inhibits the toxic action of HCl and acetic acid of pH 3.8, while Na acetate renders acetic acid of pH 5.0 already toxic when the concentration of this salt exceeds $M/32$. This toxicity is due to the non-dissociated acetic acid which is the greater the higher the concentration of Na acetate.

of HCl and $M/4$ NaCl in the case of acetic acid. This could be explained on the assumption that the NaCl prevents the diffusion of the dissociated acid in both cases but does not prevent the diffusion of the non-dissociated molecules of acetic acid to the same degree, if at all. HCl solution of pH 3.8 has less than $N/8,000$ titrable acid, while an acetic acid solution of pH 3.8 has about ten times as much titrable acid, nine-tenths of which is not dissociated.

To test further the idea that salts only inhibit the diffusion of dissociated but not of non-dissociated acid, experiments were made with mixtures of acetic acid of pH 5.0 and Na acetate. Acetic acid of pH 5.0 is harmless for the egg, as Fig. 6 shows. It was ascertained that $m/32$ to $m/4$ solutions of Na acetate were also harmless for the egg of *Fundulus* at pH 5.8. Acetic acid of pH 5.0, however, kills all the eggs rapidly if it is made up in Na acetate of $m/16$ or above. The reason is that to give a mixture of $m/16$ Na acetate and acetic acid a pH of 5.0, a concentration of between $m/64$ and $m/32$ acetic acid is required. The diffusion of non-dissociated acetic acid into the eggs killed the embryos at as low a pH as 5.0, at which strong acids like HCl or H_2SO_4 are quite harmless for the egg of *Fundulus*.

The fact that salts like NaCl can prevent the diffusion of dissociated acid but cannot prevent the diffusion of non-dissociated acid into the egg of *Fundulus*, seemed of such physiological importance as to make further proof more desirable. This was done in the experiments recorded in Fig. 8. The abscissæ in this figure are the pH while the ordinates are the percentage of embryos killed by the acid in about 14 hours. Curve II in Fig. 8 shows that when acetic acid is made up in $m/1,024$ $LaCl_3$, the eggs are not killed until the pH is below 3.5; while Curve III shows that in acetic acid alone the eggs are already killed when the pH is below 4.3. This difference is considerable and shows that $LaCl_3$ has a great retarding effect on the diffusion of dissociated acetic acid. This retarding effect of $LaCl_3$ on acetic acid is, however, less than on HCl, since Curve I shows that $LaCl_3$ inhibits the effect of the strong acid HCl much more effectively than it does the weak acetic acid. The difference is due to the fact that the $LaCl_3$ retards the diffusion of dissociated acid, HCl or acetic, but not of the non-dissociated pseudo-acid, CH_3COOH . This is made certain by Curves IV and V. Curve IV shows that in a mixture of acetic acid and $m/16$ Na acetate the eggs were killed at a pH of 5.1, and Curve V shows that this mixture is only a little less toxic in the presence of $m/1,024$ $LaCl_3$. The slight antagonistic effect revealed by a comparison of Curves IV and V is due to the inhibitory effect of $LaCl_3$ on the diffusion of the dissociated acid into the egg. The experiment was repeated with $m/64$ $CaCl_2$ instead of $LaCl_3$ and the result was the same.

By mixing acetic acid with $M/16$ Na acetate the equilibrium is shifted in favor of non-dissociated acetic acid, and $M/64$ acetic acid is required to bring a solution of $M/16$ Na acetate to a pH of 5.1. The high concentration of undissociated acetic acid killed the eggs and the experiments all proved that the rate of diffusion of this

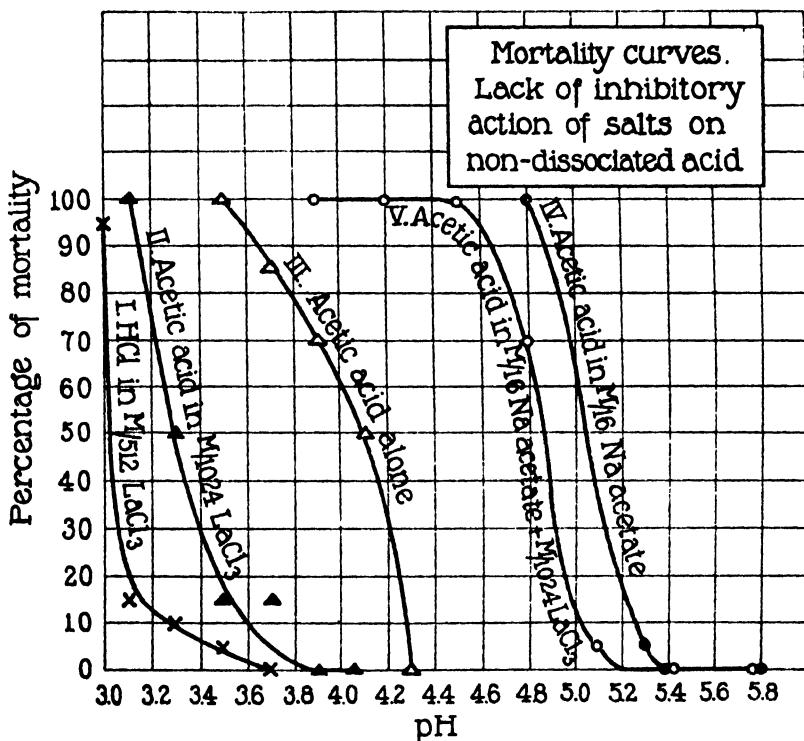


FIG. 8. Proving that $LaCl_3$ only inhibits the diffusion of dissociated acid into the egg but does not inhibit the diffusion of the non-dissociated acetic acid.

non-dissociated acid cannot be inhibited at all or only to a negligible extent by the presence of salt.

We therefore see that while the proper concentrations of salts like $LaCl_3$, $CaCl_2$, and $NaCl$ prevent the diffusion of electrolytically dissociated acid through the membranes of *Fundulus*, they will not prevent the diffusion of non-dissociated acetic acid—the so called pseudo-acid CH_3COOH . It is needless to say that this difference in the

action of salts on dissociated and non-dissociated (or pseudo-) acids must be of importance for the selective diffusion of acids through the kidneys.

In the following table is shown the concentration of acetic acid required to bring a $M/16$ solution of Na acetate to different pH. The pH was practically the same when $M/1,024$ $LaCl_3$ was in the solution.

M/16 Na Acetate in Different Concentrations of Acetic Acid.

Concentration of acetic acid..	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
pH.....	3.6	4.0	4.2	4.5	4.8	5.1	5.4	5.8

III.

The Acceleration of the Diffusion of Alkali into the Fundulus Egg by Salts.

The writer had already published the fact that either NaOH or ethylamine kills the embryo of *Fundulus* more rapidly when a salt is added.¹² It was intended to find out whether $CaCl_2$ and NaCl acted alike in this case. Fig. 9 gives the mortality curves for *Fundulus* eggs after 15 hours in $N/250$ NaOH in different concentrations of $CaCl_2$ or NaCl. It is obvious that the eggs can live in this concentration of NaOH when little or no salt is present, but that they are killed in 15 hours when the concentration of salt exceeds a certain low limit. The lowest concentration of $CaCl_2$ in which the eggs can live more than 15 hours at $24^\circ C$. is $M/1,024$, while the corresponding minimal concentration of NaCl is $M/256$.

Fig. 10 was intended to compare the effect of Na_2SO_4 and NaCl for a $N/300$ solution of NaOH. There is little difference between NaCl and Na_2SO_4 , and less between NaCl and $BaCl_2$ than between NaCl and $CaCl_2$ in Fig. 9. In all cases there is an acceleration of the diffusion of alkali into the cell due to salt.

What interests us is the fact that NaCl and $CaCl_2$ act alike on the rate of diffusion of alkali into the egg with this difference only that $CaCl_2$ is more effective than NaCl in accelerating the rate of diffusion of NaOH through the membrane of the egg of *Fundulus*.

¹² Loeb, J., *J. Biol. Chem.*, 1917, xxxii, 147.

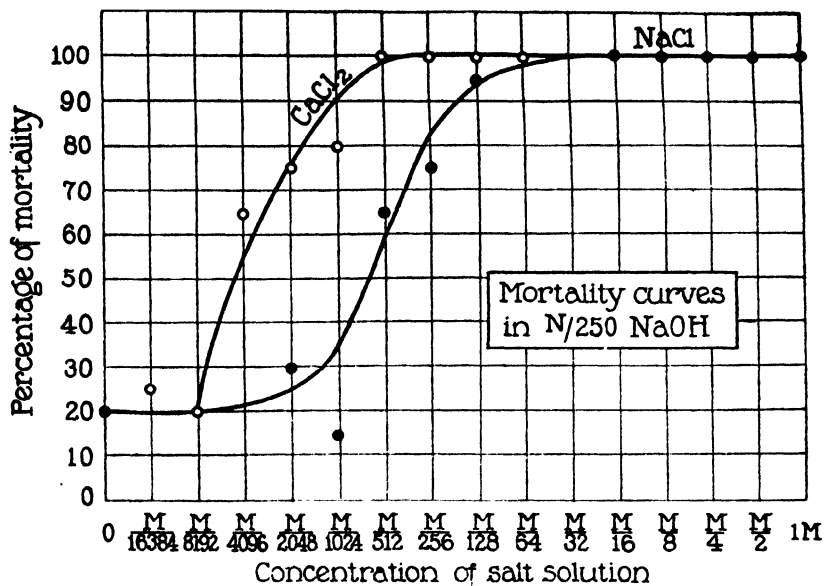


FIG. 9. $CaCl_2$ and NaCl accelerate the rate of diffusion of NaOH into the egg, $CaCl_2$ being more effective than NaCl.

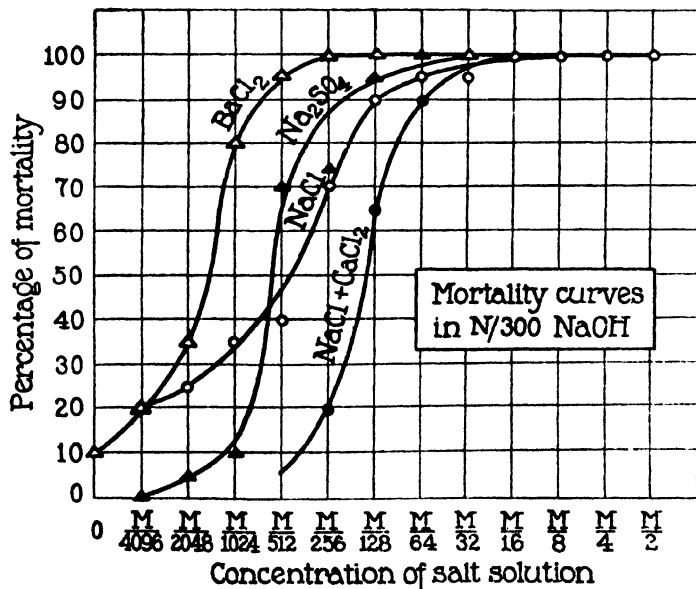


FIG. 10. Accelerating influence of different salts on the diffusion of NaOH into the egg.

IV.

The Influence of NaCl on the Diffusion of KCl through Membranes.

While NaCl and CaCl₂ behave alike in their influence on the diffusion of acid and alkali, they differ strikingly in their influence on the diffusion of KCl through the cell wall. The writer is inclined to believe that this fact may have an effect in maintaining the equilibrium of KCl inside the cells. Cells are rich in KCl and poor in NaCl, while the reverse is true for the blood serum and the tissue fluids. In 1911 Wasteneys and the writer made the observation that *Fundulus* could live indefinitely in a low concentration of KCl, while when a slight amount of NaCl was added the fish died rapidly; when more NaCl was added the fish again lived indefinitely. A slight amount of NaCl therefore accelerated the diffusion of KCl into the egg while a higher concentration of NaCl had the opposite effect, retarding the rate of diffusion of KCl into the fish.¹³

Similar results were observed by Cattell and the writer on the egg of *Fundulus*.¹⁴ A concentration of KCl which alone was not able to cause the heart to stop beating was rendered efficient when a little NaCl was added, while with the addition of more NaCl the diffusion of KCl into the egg was blocked again.

When embryos whose hearts had been caused to stop beating in a KCl solution were put into a NaCl solution (or solutions of certain other salts), the heart beat was resumed again after some time; but when the eggs poisoned by KCl were put into distilled water or a solution of a non-electrolyte, such as cane-sugar or glucose or glycerol or urea, the hearts would not recover. These embryos have the peculiarity that they can survive for as long as a week or more without heart beat. It was found that such eggs which had failed to recover from the KCl in distilled water or sugar solutions for several days would recover promptly when put into the solution of the proper salts. All these experiments proved that the diffusion of KCl through the membrane of the egg was only possible when the egg was in a solution of a sodium salt or when the membrane contained some such

¹³ Loeb J., and Wasteneys, H., *Biochem. Z.*, 1911, **xxxi**, 450; **xxxii**, 155.

¹⁴ Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, **xxiii**, 41. Loeb, J., *J. Biol. Chem.* 1916, **xxvii**, 339, 353, 363.

salts. This salt effect required for the diffusion of KCl through the membrane increased for sodium salts with the valency of the anion. The reader is referred for further details to the previous publications.¹⁸

In these older experiments one question was left undecided; namely, whether or not CaCl_2 acts like NaCl on the rate of diffusion of KCl through the membrane of the egg. In order to obtain a definite answer a special method must be used. Eggs of *Fundulus* in which the circulation was developed were taken from sea water and put for 48 hours into a $\text{M}/2$ KCl solution of pH about 5.8 at a temperature of about 24°C . The purpose was to fill the eggs with an excessive dose of KCl so that the recovery should not be too quick. This precaution had been neglected in the writer's previous experiments and as a consequence recoveries occurred for which the outside solution was not always responsible. It was assumed that the recovery, *i.e.* the resumption of the heart beat, occurred when the excess of KCl had diffused out of the egg. The eggs thus poisoned with KCl were then transferred into distilled water for 2 days to remove all salts (adhering to the outer surface of the egg membrane). None of the hearts recovered in the distilled water. The eggs were then put into different concentrations of different salts all of a pH of 5.8 (*i.e.* the pH of the distilled water) and examined after certain intervals. Twenty eggs were always put into 50 cc. of the salt solution. Fig. 11 gives the curves for the recovery of eggs in 3 days in solutions of NaCl, CaCl_2 , and LaCl_3 . The abscissæ are the concentrations of the salt solutions, the ordinates the percentage of eggs that had recovered. It is obvious that recovery occurred rapidly in NaCl, while the recovery was very slow in CaCl_2 and while practically no recovery occurred in LaCl_3 . It was not due to any injurious action of CaCl_2 that the eggs did not recover since it was found in control experiments that eggs which had failed to recover after 2 or 3 days in, *e.g.*, a $\text{M}/64$ solution of CaCl_2 , recovered in less than a day when put into a solution of $\text{M}/64$ $\text{Na}_4\text{Fe}(\text{CN})_6$ or some other sodium salt.

Since in sea water and in blood NaCl is always accompanied by CaCl_2 in the proportion of approximately 100 molecules of NaCl to 1.5 molecules of CaCl_2 , it seemed of importance to determine the influence of different concentrations of such mixtures of NaCl + CaCl_2 on the rate of diffusion of KCl through the membrane. These experiments

yielded the interesting results that the balanced solution of NaCl + CaCl₂ accelerated the diffusion most at a concentration of $M/4$ and $M/2$ while at higher concentrations the influence became less (Curve I, Fig. 12). This was not due to any injury to the egg but corresponded to the earlier results that when the concentration of NaCl exceeds a certain limit it retards the diffusion of KCl through

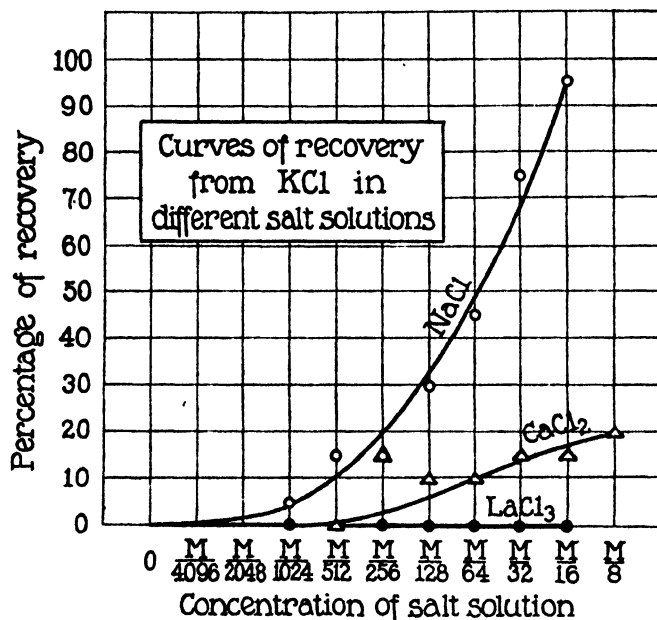


FIG. 11. Influence of various salts on the rate of recovery of eggs from an excess of KCl. Abscissæ are the concentrations of salts; ordinates, percentage of eggs which have recovered from KCl. Recovery is most rapid in NaCl, less rapid in CaCl₂, and least rapid in LaCl₃. pH 5.8.

the membrane. That the higher concentration of NaCl + CaCl₂ only retarded the rate of diffusion of KCl without injuring the egg follows from the fact that 24 hours later more eggs had recovered in these higher concentrations than had after 20 hours (Curve II, Fig. 12).

It had already been noticed in the older experiments that the Na salts caused the recovery of the eggs the more promptly the higher

the valency of the anion of the salt. Since in these experiments no attention had been given to the pH of the solution, it was necessary to repeat the experiments. In the new experiments the salt solutions always were made up for the same pH; namely, 5.8. It is obvious that the recovery is the most rapid in $\text{Na}_4\text{Fe}(\text{CN})_6$, next in Na_2SO_4 , and next in NaCl , while it is slowest in MgCl_2 (Fig. 13).

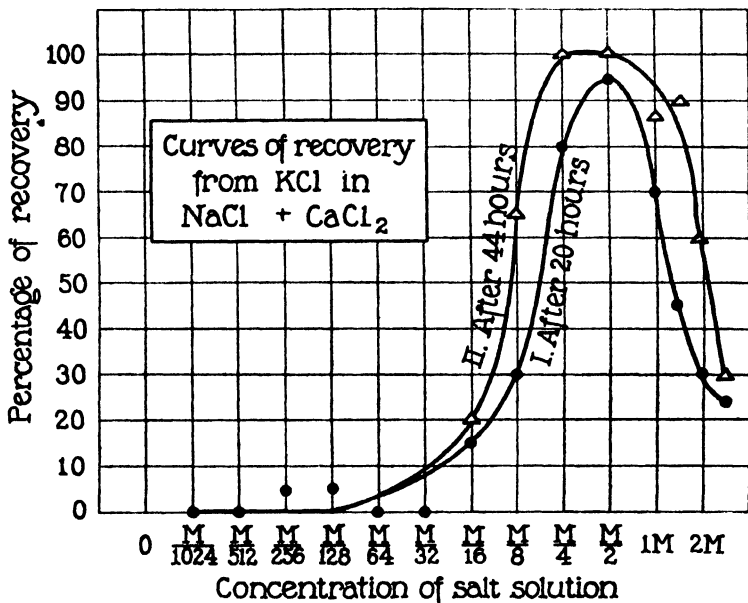


FIG. 12. Influence of concentration of $\text{NaCl} + \text{CaCl}_2$ (balanced solution) on recovery of eggs from KCl poisoning, showing that when the concentration of salt increases from $\text{M}/128$ to $\text{M}/2$ the rate of recovery increases, while after that the rate of recovery diminishes with further increase in concentration.

It may incidentally be stated that the recovery as a rule was a little quicker in MgCl_2 or BaCl_2 than in CaCl_2 , while MnCl_2 and SrCl_2 acted very much like CaCl_2 . In LiCl and NH_4Cl the recovery was decidedly slower than in NaCl (Fig. 14).

Entirely similar results as those described for the rate of diffusion of KCl were obtained in regard to the influence of these salts on the rate of diffusion of Rb through the membrane of the egg of *Fundulus*.

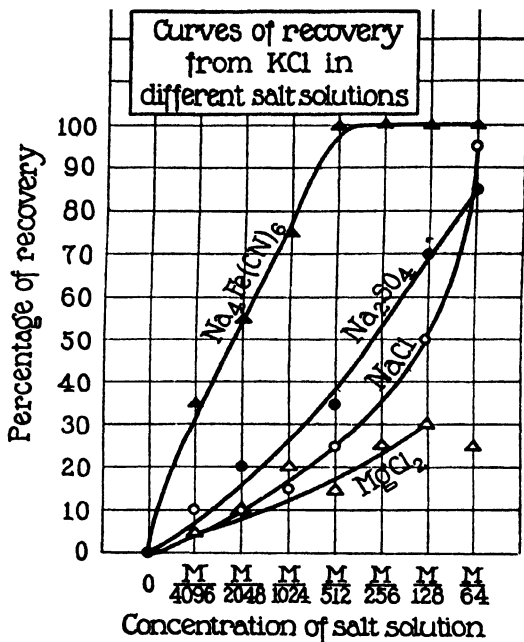


FIG. 13. Influence of different salts on the rate of recovery. The rate increases for Na salts with the increase in valency of the anion. pH 5.8.

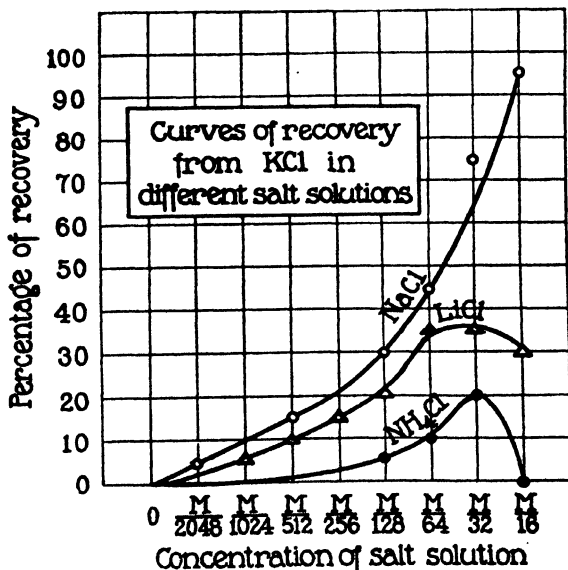


FIG. 14. Influence of NaCl, LiCl, and NH_4Cl on the rate of recovery from KCl. Influence of NaCl is greater than that of LiCl or NH_4Cl .

THEORETICAL REMARKS.

It has been customary to explain phenomena of selective diffusion through membranes as well as differences in the relative concentration of ions between cells and blood by the assumption that the diffusion depends on the lipoids of the cells and that only substances that are soluble in these lipoids can diffuse through the cell walls. Thus K was considered lipid-soluble, and Na and Ca were not considered lipid-soluble. These ideas are purely speculative and the best that can be said for them is that they are not contradicted by the facts and that possibly at some future date these guesses may turn out to have been correct. The facts enumerated in this paper point, however, to a definite influence of NaCl and CaCl₂ on selective diffusion and these facts exist and are of importance regardless of the ultimate explanation of the mechanism by which salts influence diffusion through the membrane of cells.

We understand why Na and Ca cannot diffuse into the cells of our body, since such a diffusion is rendered impossible by both the CaCl₂ and the NaCl present in blood, which seem to render the membrane of most kinds of cells impermeable for Na as well as Ca. We see, on the other hand, that the NaCl must favor the diffusion of KCl into the membrane, and this favorable effect is not counteracted by the Ca, as Fig. 12 shows. If it could be shown that NaCl + CaCl₂ favor also the rate of diffusion of Cl ions through the membrane, it would follow that KCl must accumulate in the cells until the molar concentration of Cl ions in the cells equals the Cl ions in the blood or tissue fluid. While this may be the case, the writer does not wish it understood that he considers this influence of NaCl upon the diffusion of KCl through the membrane as the sole cause of the unequal distribution of K and Na in cells and blood. That such a suggestion would be premature is indicated by the work of Loeb, Atchley, and Palmer.¹⁵ It is certain, however, that the effect of NaCl + CaCl₂ upon the rate of diffusion of KCl through the membrane must influence the ease with which this ionic equilibrium between blood and cells is maintained.

¹⁵ Loeb, R. F., Atchley, D. W., and Palmer, W. W., *J. Gen. Physiol.*, 1921-22, iv, 591.

The fact that salts accelerate the rate of dissociated alkalies and retard the rate of diffusion of dissociated acids, while they have no retarding influence on the rate of diffusion of non-dissociated acid (and, perhaps, also non-dissociated alkali) has probably some bearing on secretions and certainly an important bearing on the use of drugs and disinfectants.¹⁶ It is also possible that the lack of inhibitory action of salts on the rate of diffusion of non-dissociated acids may ultimately furnish a clew for the understanding of the mechanism by which salts modify the rate of diffusion of ions through the membrane.

Finally the writer calls attention to the fact that salts accelerate the rate of diffusion of acids through collodion membranes¹⁷ while they have the opposite effect on the rate of diffusion of acids through the membranes of *Fundulus*. This must be due to the fact that the *Fundulus* membrane or the membranes of cells in general differ from collodion membranes in a chemical or physical way.

It may also be stated that acid diffused more rapidly into eggs which had been killed by keeping them for 5 minutes at a temperature of 80°C., but that the rate of diffusion of acid into such eggs is also retarded by salts.

SUMMARY AND CONCLUSIONS.

1. It is shown that NaCl acts like CaCl₂ or LaCl₃ in preventing the diffusion of strong acids through the membrane of the egg of *Fundulus* with this difference only that a M/8 solution of NaCl acts like a M/1,000 solution of CaCl₂ and like a M/30,000 solution of LaCl₃.

2. It is shown that these salts inhibit the diffusion of non-dissociated weak acid through the membrane of the *Fundulus* egg but slightly if at all.

3. Both NaCl and CaCl₂ accelerate the diffusion of dissociated strong alkali through the egg membrane of *Fundulus* and CaCl₂ is more efficient in this respect than NaCl.

4. It is shown that in moderate concentrations NaCl accelerates the rate of diffusion of KCl through the membrane of the egg of *Fundulus* while CaCl₂ does not.

¹⁶ Michaelis, L., and Dernby, K. G., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 194.

¹⁷ Loeb, J., *J. Gen. Physiol.*, 1922-23, v, 255.

THE INFLUENCE OF SALTS ON THE RATE OF DIFFUSION OF ACID THROUGH COLLODION MEMBRANES.

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(Received for publication, September 5, 1922.)

When a solution of a salt, *e.g.*, CeCl_3 or CaCl_2 or NaCl , in HCl , with a pH of 3.0, is separated by a collodion membrane from a pure solution of HCl of the same pH, salt will diffuse out until the solution on both sides of the membrane is identical. Since the concentration of HCl measured by the hydrogen electrode is the same on opposite sides of the membrane at the beginning of the experiment, one would expect that no change in the pH should occur during the experiment. The writer found, however, that at first acid is rapidly driven by the salt to the other side of the membrane, so that at first the concentration of the acid is diminished on the side of the salt solution and increased on that side of the membrane where the solution was originally free from salt.¹ This occurs, of course, only during the first hours of the experiment. When the concentration of the salt has become the same on the opposite sides of the membrane, the pH also becomes the same again on both sides of the membrane.

The following experiments may serve as an illustration. Collodion bags, treated with gelatin, of about 50 cc. contents, were filled with a $m/256$ solution of a salt made up to pH 3.0 through the addition of HCl . These bags dipped into beakers filled with 350 cc. of HCl of pH 3.0 (about $N/1,000$) but without salt. After different intervals the experiment was terminated and the pH inside and outside was determined. It was found that after an hour the pH inside had increased considerably (*i.e.* the concentration of hydrogen ions in the salt solution had diminished), while outside the change of pH was small on account of the fact that the volume of solution was seven times as great as inside the bag. Table I gives some of the results. In Experiments 1 to 4 the collodion membrane was coated with gelatin, while in Experi-

¹ Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 213.

ment 5 the membrane was not treated. The phenomenon is observed in both cases. The initial concentration of the salt inside the bag was always $M/256$, but, of course, diminished during the experiment.

TABLE I.

Change of Hydrogen Ion Concentration on Opposite Sides of the Membrane during Period of Diffusion of Salts.

Time, hrs.		0	1	2	3	5	6	8	22
1. $M/256$ $CeCl_3$.	pH inside.....	3.01	3.29	3.29	3.32	3.17	3.20	3.16	3.04
	pH outside.....	2.97	2.97	2.95	2.95	2.97	2.98	2.97	2.99
	Difference.....	0.04	0.32	0.34	0.37	0.20	0.22	0.19	0.05
2. $M/256$ $CaCl_2$.	pH inside.....	2.99	3.26	3.26	3.21	3.20	3.21	3.10	3.02
	pH outside.....	2.97	2.97	2.97	2.96	2.98	2.98	2.98	2.99
	Difference.....	0.02	0.29	0.29	0.25	0.22	0.23	0.12	0.03
3. $M/256$ $NaCl$.	pH inside.....	2.98	3.13	3.12	3.09	3.05	3.05	3.00	
	pH outside.....	2.98	2.98	2.99	2.98	2.99	3.00	2.99	
	Difference.....	0	0.15	0.13	0.11	0.06	0.05	0.01	
4. $M/256$ Na_2SO_4 .	pH inside.....	3.06	3.08	3.01	3.01	3.01	3.01	3.05	
	pH outside.....	2.98	3.01	3.00	3.01	3.00	3.02	3.03	
	Difference.....	0.08	0.07	0.01	0	0.01	0.01	0.02	
5. $M/256$ $CaCl_2$. (Membrane not coated with gelatin.)	pH inside.....	2.98	3.11	3.11	3.08	3.04	3.04	3.02	
	pH outside.....	2.98	2.95	2.97	2.99	2.97	2.97	2.99	
	Difference.....	0	0.16	0.14	0.09	0.07	0.07	0.03	

These experiments show, first, that the expulsion of acid from the salt solution into the solution free from salt during the first hours of the experiment is in the case of chlorides the more considerable the higher the valency of the cation; in other words, it increases with the concentration of Cl ions in the solution. Thus after 3 hours the difference in pH inside and outside is 0.37 for $CeCl_3$, 0.25 for $CaCl_2$, and 0.11 for $NaCl$. This expulsion of acid occurs regardless of the fact whether or not the membrane is coated with gelatin, as a comparison of Experiment 2 with Experiment 5 shows. After 3 hours the difference in acid on the opposite sides of the membrane becomes less and finally disappears. In the case of Na_2SO_4 this effect is not noticeable.

When the writer first observed these phenomena he was under the impression that they were due to the action of the membrane, and he expressed this belief in a paper published a year ago.¹ Recent experiments to be mentioned presently have convinced him that the membrane has little if anything to do with this phenomenon and that it finds its explanation in the influence of salts on the diffusion constant of acids discovered by Arrhenius and described by him as follows:

"The theoretical study of the phenomenon of diffusion has led to a conclusion which seems very paradoxical. If hydrochloric acid diffuses in water, its diffusion constant is found to be 2.09 at 12°C., which also agrees very well with the theory of Nernst. If instead of using pure water for the diffusion, I take a solution of sodium chloride, I might expect that the molecules of HCl moved, *i.e.*, diffused more slowly in that medium than in water because of the increase of the viscosity on addition of NaCl. But instead of that an increase of the constant of diffusion is observed. For instance into a cylindrical vessel was poured a layer of 1 cm. height of 1.04 *n* HCl and over it was placed pure water to a height of 3 cm. The diffusion constant was found to be 2.09 at 12°C. In another experiment 0.1 *n* NaCl was used instead of water, so that at the bottom of the cylindrical vessel was a 1 cm. high layer 1.04 *n* in regard to HCl and 0.1 *n* in regard to NaCl and over it were placed 3 cm. of 0.1 *n* NaCl.

"The diffusion constant was now 2.50. According to Nernst's theory I calculated 2.43. In 0.67 *n* NaCl the constant was still higher 3.51, calculated 3.47. Many analogous experiments with results agreeing with the dissociation theory were performed with nitric and hydrochloric acid, caustic potash and soda.

"The explanation is that when the H-ions (*i.e.*, the acid) diffuse in pure water they must drag the (about 5 times) more immobile Cl-ions with them in equivalent number. If now Na-ions are distributed in the same fluid, these ions are carried back in the opposite direction of the diffusing H-ions because of the electric forces which hold back the H-ions and pull on the Cl-ions in the direction of diffusion (upwards in the experiments). The driving back of the Na-ions partly neutralizes these electric forces, so that therefore the H-ions are not so strongly held back nor the Cl-ions pushed up as in pure water. Therefore the H-ions diffuse more rapidly and that in a so much higher degree as the Na-ions are more numerous relatively to the H-ions. The maximal velocity which the H-ions may reach at 12° is that corresponding to no hindrance from electrical forces and gives a diffusion constant 6.17. With 0.52 *n* HCl and 3.43 *n* NH₄Cl I reached a value of 4.67 instead of theoretically calculated 5.72.—It must be borne in mind that at these high concentrations the degree of dissociation of the HCl is diminished, which is not taken into consideration, and therefore the observed dissociation constant is smaller than the calculated one determined with the supposition that the electrolytic dissociation is complete.

"This phenomenon is a so-called salt action, which can not be explained if we suppose the molecules of the diffusing acids or bases and the added salts not to be dissociated into electrically charged ions. It is a real proof of the electrolytic dissociation hypothesis."²

On the basis of Arrhenius' statement, the phenomenon observed by the writer might find its explanation in the following way. When a solution of, *e.g.*, $M/1,000$ CeCl_3 is made up in HCl so that the pH is 3.0, and if this solution is put into a collodion bag of 50 cc. volume, which is immersed in a beaker containing pure HCl (without salt) also of pH 3.0, the osmotic pressure of Cl ions is about four times greater in the salt solution than on the opposite side of the membrane. Hence four times as many Cl ions will diffuse through the membrane from the salt solution into the water as will diffuse simultaneously in the opposite direction. H and Ce ions will be dragged along electrostatically with the moving Cl ions, but on account of their greater mobility H ions will follow the Cl ions more readily than will the slow Ce ions. As a consequence the hydrogen ion concentration will rise on the water side of the membrane and diminish on the side of the salt solution; but on account of the fact that the volume of water was seven times as great as that of the salt solution inside the collodion bag, the change in pH was more marked in the salt solution inside the bag than outside. If this explanation is correct, the membrane itself has nothing to do with the phenomenon except to make it easier to demonstrate the salt effect on the rate of diffusion of the acid. If this conclusion is correct, it should be possible to prove that the rate of diffusion of acid into water through a membrane is accelerated through the addition of salt and the more the higher the concentration of the salt.

This proof was furnished in the following way. 350 cc. of different concentrations of NaCl , CaCl_2 , LaCl_3 , or Na_2SO_4 were made up in HCl so that each solution was $N/2,000$ in regard to the acid. Into these mixtures of $N/2,000$ HCl and salt were dipped collodion bags with a volume of about 50 cc. filled with water of pH 4.7. In this case the salt solution was outside the bag and the water free from salt was

² Arrhenius, S., *Theories of solutions*, New Haven, London, Oxford, 1912, 163-165.

inside the collodion bag. Both salt and acid diffused into the bag. After 20 minutes the collodion bags were taken out, the volume of liquid measured, and 10 cc. of the solution were titrated with $N/400$ NaOH to pH 5.4, using methyl red as an indicator.

In these experiments the concentration of the acid outside did not change materially since the volume of the outside solution was seven times as great as the inside solution, and the volume of the liquid inside the bag also remained practically the same except at the higher concentrations of the salt where the osmotic effect became noticeable. We may therefore use the titration value for 10 cc. of the content of the bag as a measure for the rate with which acid diffuses from the outside solution into the collodion bag. These titration values are plotted in Fig. 1 as ordinates over the concentrations of the salts as abscissæ. 0.25 cc. of $N/400$ NaOH were required to bring the acid contained in 10 cc. of the contents of the collodion bag to pH 5.4 when no salt was present. If we call this rate for the diffusion of acid through the membrane 1, then it is seen that the rate of diffusion of HCl is doubled when the concentration of NaCl is between $M/64$ and $M/128$, when the concentration of $CaCl_2$ is between $M/128$ and $M/256$, and when the concentration of $LaCl_3$ is about $M/512$ or perhaps a little below. It is obvious that the effect of these three salts on the rate of diffusion of HCl through the membrane increases with the concentration of the Cl ions and that all three salts accelerate the rate of diffusion of HCl through the membrane. H_2SO_4 diffuses more slowly through the membrane than HCl, since the titration value for acid without salt is 0.20. This rate of diffusion is doubled when the concentration of Na_2SO_4 is between $M/256$ and $M/128$. At higher concentrations both Na_2SO_4 and $CaCl_2$ show anomalies which need not be discussed in this connection.

These experiments were open to the objection that the pH of the $N/2,000$ HCl solution was different in $LaCl_3$, $CaCl_2$, or NaCl and that this caused the difference in the influence of the three salts on the rate of diffusion. Measurements of the pH showed that it was practically the same in all three solutions and had therefore nothing to do with the result. This was, however, not true in the case of mixtures of H_2SO_4 and Na_2SO_4 (as had already been found by Thomas and Baldwin*), and for high concentrations of $CaCl_2$. These interesting phenomena can, however, not be discussed in this connection.

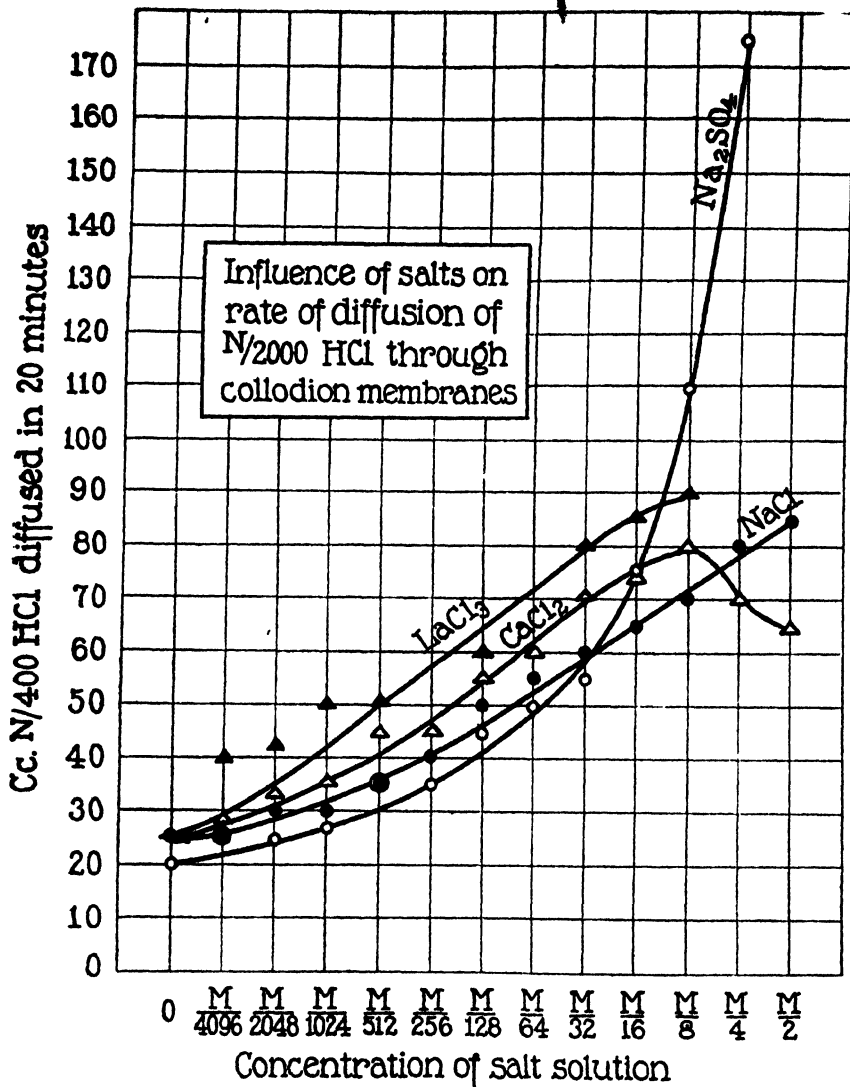


FIG. 1. Influence of salts on rate of diffusion of N/2,000 HCl through collodion membranes. Abscissæ are the concentrations of salts; ordinates, cubic centimeters of N/400 NaOH required to bring 10 cc. of water inside to pH 5.4 after 20 minutes of diffusion.

1 a series of papers and a recent book the writer has tried to prove that the influence of electrolytes on the colloidal properties of protein solutions, such as osmotic pressure, viscosity, membrane potentials, is merely the consequence of the Donnan equilibrium. The equilibrium rests on the fact that many membranes which are freely permeable for small crystalloidal ions are impermeable for protein ions. The writer has shown that proteins behave in regard to chemical reactions like crystalloids and the same seems to be true as far as solubility is concerned. This then leads to the conclusion that the only reason for the colloidal behavior of protein solutions in regard to osmotic pressure, viscosity, etc., lies in the non-diffusibility of proteins through membranes and gels which are permeable to crystalloids. If we could produce membranes permeable to Na but not to SO_4 ions it would be found that a Na_2SO_4 solution would show colloidal behavior in regard to osmotic pressure and membrane potentials. This postulate of the theory of colloidal behavior is met in the facts published in this paper. It is not necessary that the membrane should be completely impermeable for one ion; it suffices that the rate of diffusion of one type of ions be considerably slower than that of another kind. The slow ions are the cations of salts, especially the polyvalent cations.

These experiments were undertaken to find out whether the influence of salts on the rate of diffusion of acid through the membranes of living cells was the same as through collodion membranes. This is not the case since salts retard the rate of diffusion of acid through membranes of living cells instead of accelerating it. This retarding effect of salt on the diffusion of acid—the so called antagonistic salt effect—was observed by Loeb and Wasteneys in the case of the marine fish *Fundulus*.⁴ Thus the fish died in 3 M/10,000 HNO_3 in less than an hour while they lived indefinitely when the same HNO_3 solution was made up in M/10 NaCl. CaCl_2 was much more effective in the prevention of the toxic effects of acid. Later the same inhibiting effect of salt on the toxic effect of acid was observed in the eggs of *Fundulus*.⁵

³ Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 1981.

⁴ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

⁵ Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii, 147.

Since the effect of salts on the diffusion constant of acids must be the same in the case of membranes of living cells as in the case of collodion membranes, the retarding effect of salts on the diffusion of acids in the egg of *Fundulus* can only be ascribed to a modification of the membranes of *Fundulus* by the salts whereby a block is created to the diffusion of acids.

SUMMARY AND CONCLUSION.

1. The writer had previously published the observation that if a salt solution made up in an acid solution (*e.g.* HCl) of a definite pH (*e.g.* 3.0) is separated by a collodion membrane from pure water containing the same acid of the same pH, acid is at first driven from the salt solution into the water, so that the pH of the latter becomes at first lower than that of the solution.
2. It is shown in this paper that this paradoxical phenomenon is not due to any peculiarity of the membrane but is a consequence of the well known fact that the diffusion constant of an acid is increased by a salt.

THE MECHANISM OF THE INFLUENCE OF ACIDS AND ALKALIES ON THE DIGESTION OF PROTEINS BY PEPSIN OR TRYPSIN.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, September 21, 1922.)

It has long been known that pepsin digestion occurs only in acid solution while trypsin digestion takes place in neutral or alkaline solutions. This marked influence of the reaction on the rate of hydrolysis is a characteristic property of enzymes in general. A great advance in the explanation of this phenomenon was made by Sørensen (1) who was able to show in the case of a number of enzymes that the controlling factor was the hydrogen ion concentration, and that the other ions present were of secondary importance. Michaelis (2) found that the curves expressing the rate of hydrolysis as a function of the pH could be accurately calculated on the assumption that the enzyme was a weak acid or base, and that the activity was due either to the ion or to the undissociated molecule, depending on the nature of the enzyme. Michaelis' experiments leave little doubt that the experimental curve is very similar to a dissociation curve. The weak point in the hypothesis is the lack of direct or independent evidence for the ionic character of the enzymes and the fact that migration experiments (3) on pure pepsin appear to contradict some of the assumptions used in the calculations. It had been suggested by Euler (4) and by Arrhenius (5) that the effect of the acid was upon the substrate rather than on the enzyme. The writer was able to show (6) that in the case of pepsin this mechanism served very well to explain the results, since it was found that the rate of hydrolysis could be quite accurately predicted from a measurement of the concentration of ionized protein and also that the pH for minimum digestion shifted with the isoelectric point of the protein. This indicates that the effect is primarily on the protein. The present paper contains

the results of experiments designed to test this assumption more fully and to extend the observations to trypsin. It has been found that the rate of digestion of gelatin, casein, and hemoglobin by pepsin or trypsin may be predicted from the amount of protein ion present. The acid salt of these proteins is rapidly attacked by pepsin, and the alkali salt by trypsin. The curve for the rate of digestion plotted as a function of the pH is therefore nearly identical with the titration (dissociation) curve of the proteins. The same result has been found with trypsin, edestin, and globin in that the alkaline titration curve of these proteins agrees with the curve for the rate of trypsin hydrolysis.

Experimental Procedure.

Regulation and Measurement of the pH.—The measurements were made with the gas chain at 33°, using 0.10 N HCl as the standard. The pH of this acid was taken as that given by the conductivity ratio: 1.036.

Buffers.—In the pepsin experiments no buffer was used; the protein solution was simply titrated to the desired pH with acid (HCl). There was no significant change in pH during the reaction. Trypsin hydrolysis causes a very large change in the pH and it is necessary to use a powerful buffer. It was found that a combination of phosphate, borate, and citrate would furnish a solution having a high buffer value over a wide range of pH. It has the further advantage that the nature of the ions does not vary. The titration curve of this buffer is given in Fig. 1. It is not intended to be used as a standard and for accurate values the solutions should be checked in some way. The final concentration of the buffer was usually one-quarter that of the original solution.

Pepsin.—The pepsin used was a sample of specially active Fairchild's pepsin (U.S.P. 1:20,000).

Trypsin.—Fairchild's trypsin was used. It was purified by dialysis, as described in a preceding paper (7).

Temperature.—All experiments were made at $33^{\circ} \pm 0.5^{\circ}\text{C}$.

Determination of the Rate of Digestion. *Pepsin. Gelatin.*—The rate of hydrolysis was determined by noting the time for a sample to reach a definite degree of liquefaction, as previously described (6).

Casein.—The casein (8) was dissolved in dilute NaOH and brought to the required pH by addition of the appropriate buffer. An aliquot portion of the solution was removed, brought to approximately pH 4.7 and added to 3 volumes of acetate buffer pH 4.7. The precipitated casein was then filtered, washed with water, dried at 100° and weighed. The curves obtained in this way were plotted and the time required to dissolve about 10 per cent of the total casein read off. This value has been taken as the reciprocal of the velocity of hydrolysis.

L. myoglobin.—The hydrolysis was followed by means of the increase in amino nitrogen, by the Van Slyke method (9).

Trypsin. Casein.—The same method was used as that described above. The hydrolysis was also followed by the Van Slyke method for amino nitrogen.

Gelatin.—The hydrolysis was followed by the increase in formol titration (10) and also by the Van Slyke method.

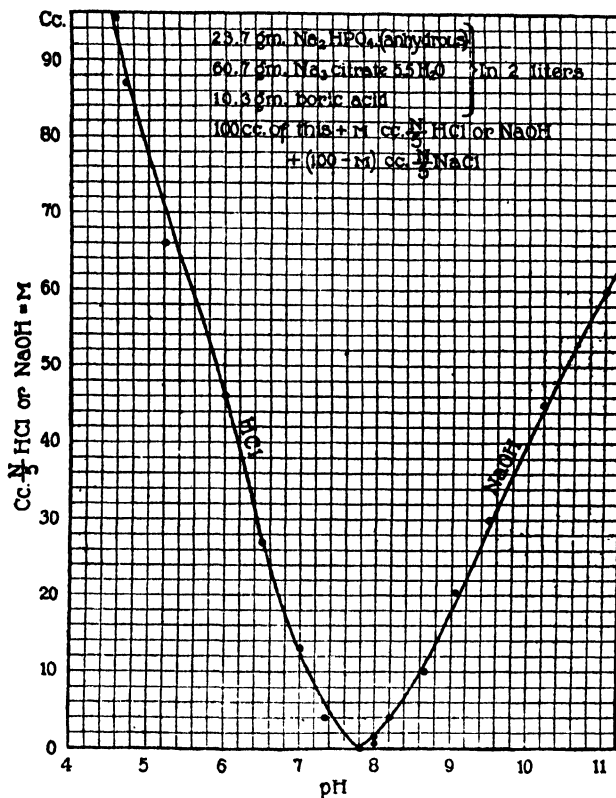


FIG. 1.

Globin.—The globin was prepared as described by Robertson (11). The rate of hydrolysis was followed by bringing the solution to the isoelectric point (about pH 6) and filtering off and weighing the undissolved protein.

Edestin.—The writer is indebted to Dr. Hitchcock for the edestin used in this experiment. The rate of hydrolysis was determined by the Van Slyke method and also by precipitating and weighing the unattacked protein.

Titration Curves.—The titration curves were determined by adding a known amount of alkali or acid to the protein solution and determining the pH. A similar series was carried out in which the protein was omitted. The amount of protein combined with the acid (or alkali) was then determined by subtracting the cubic centimeters of acid required to bring water to any given pH from the cubic centimeters of acid required to bring an equal volume of the protein solution to this pH. This correction is negligible between pH 3 and 9. The curves obtained were identical with those given by Loeb.

In the case of casein solution it was found that two entirely different pH curves were obtained (in the region near the isoelectric point), depending on whether the supernatant fluid or the whole suspension was measured. This behavior had also been noted by Cohn. If the measurement was made on the whole suspension, the curve rises much more steeply near the isoelectric point and is similar to the curve given by Van Slyke and Baker (8). It also resembles closely the curve for gelatin and does not conform to the typical titration curve. Loeb (12) has recently shown that this effect is due to the fact that the pH of a suspension is intermediate between that of the supernatant liquid and that of the particle. The correct curve is that of the supernatant fluid.

Conductivity measurements (6) show that the protein salt is completely dissociated at least until a very large excess of the common ion is added. The titration curve, therefore, gives directly the amount of ionized protein up to the maximum value; beyond this point the amount of ionized protein decreases whereas the amount of combined acid remains constant. This decrease in the ionized protein can be followed in the case of gelatin (on the acid side) as described in a previous paper (6). In the case of the other proteins, however, the point lies in the region of such strong acid that the measurement is very uncertain, owing to the very large value of the correction for the free acid. The determination of this value was, therefore, not attempted in the case of the other proteins. On the alkaline side the conditions are more complicated, owing to the fact that there are apparently two alkaline salts formed, *i.e.*, there is a group of acid radicals that is completely neutralized at pH 9 and another group that is not neutralized until a pH of 11 or 12 is reached. This is very marked in the case of gelatin and casein. Hemoglobin, however, shows only a very slight flattening of the curve at pH 7.5; the second group of acid radicals evidently begins to combine with the alkali before the first group is completely neutralized.

Effect of the Amount of Enzyme.—It has been found by the writer (13) and also by Ringer (14) that trypsin is very rapidly destroyed in solutions of pH greater than 10. In order to reduce this effect, it is necessary to use relatively large amounts of trypsin so that the experiment is completed in as short a time as is permitted by the experimental limitations. If this is not done the results are complicated by the influence of the pH on the trypsin itself. This effect would evidently cause the optimum to shift to the acid side and would be more marked the more dilute the trypsin; *i.e.*, the longer the experiment is allowed to run. Several experiments were made to test this point and it was found that, with the amounts of trypsin used in these experiments, the position of the optimum was independent of the amount of trypsin used. The drop in the rate of digestion in strong alkali, therefore, cannot be attributed to the destruction of the trypsin by the alkali. This complication does not enter in the case of pepsin, since this enzyme is stable, even in strong acid.

Presentation of Results.—For the sake of brevity the original curves obtained for the increase in the digestion with time are not given, but only the rates interpolated from these curves. The results were plotted on a large scale and the time (in hours) required to hydrolyze 10, 20, and 30 per cent of the protein read off from the curves. The reciprocal of these times is taken as a measure of the rate of digestion. It was found that the values obtained in this way were the same, within the rather large experimental error, independent of the percentage hydrolysis which was used as the end-point. In other words, the rate of hydrolysis-pH curve is independent of the stage of hydrolysis, in the beginning. If the results are taken from a much later stage of hydrolysis, the curve is changed slightly, as would be expected, since the secondary reactions become more predominant and the effect of the pH on the destruction of the enzyme more pronounced. No significant effect of different enzyme concentration could be noted, nor did the different methods of following the hydrolysis give results which differed more than the experimental error. It is unnecessary, therefore, to distinguish between the different experiments.

In order to reduce all the experiments to a comparative basis, the results have been calculated to a percentage scale; *i.e.*, the maximum rate of hydrolysis has been taken as 100 and the rates at other pH expressed as percentages of this maximum.

Comparison of the Titration Curve and the Rate of Hydrolysis.—In order to compare the rate of hydrolysis with the amount of protein salt found, it is necessary to express both quantities in the same units. This has been done by calculating the titration curve also to a percentage basis. The largest amount of alkali or acid which is found to combine with the protein has been taken as 100 and the amounts at other pH expressed as per cent of this figure. The maximum on the acid side is well defined and there is no difficulty in deciding at which pH the protein is all present as the acid salt. On the alkaline side, as mentioned above, the titration curve shows that there are two alkaline salts formed. It could not be told *a priori* from the titration curve alone which of the salts was attacked by the trypsin. Comparison with the hydrolysis pH curve, however, shows that, except in the case of globin, the maximum rate of hydrolysis occurs at the same pH as does the maximum formation of the *first* alkali salt. The point at which the greatest amount of this salt is formed has therefore been taken as 100. In the case of globin the hydrolysis increases with the second rise in the titration curve, indicating that in this case the second salt formed is still more easily attacked. The second maximum in the titration curve has therefore been taken as 100 in the case of this protein. The position of this maximum is much more difficult to determine and it is possible that the quantitative agreement of the titration and rate of hydrolysis curves of globin is partly due to an incorrect value for the maximum point of the titration curve. There is no doubt, however, that the curves agree qualitatively in that they both have a flat portion between pH 8 to 9, followed by a second rise; the only uncertainty is as to the exact coincidence of this second maximum. The hydrolysis curve cannot be carried further, owing to the destruction of the trypsin.

Results of the Experiments. Influence of the Ions Other Than H and OH and the Influence of the Physical Properties of the Substrate.—The work of Sørensen (1), Michaelis and his coworkers (2), and of the writer (6, 13, 15) has shown conclusively that the hydrogen or

hydroxyl ion is the determining factor and that the nature and valence of the other ion is of very minor importance as is the physical condition of the substrate. Nevertheless the question of the influence of the "degree of hydration" or swelling of the protein has again been raised by Ostwald (16). This idea may be traced back to Brücke (1859). Brücke (17) was a pioneer worker and it is easy to understand his suggestion that swelling and digestion are connected since both increase with increasing amounts of acid. At present, however, it would seem to be difficult to add anything to the proof already existent that the swelling, osmotic pressure, "degree of hydration," etc., have no direct connection with the hydrolysis of proteins by either pepsin or trypsin. Since, however, this is a fundamental point in the study of these enzymes, some of the experiments referred to were repeated. The critical experiment is to compare the rate of hydrolysis of a protein in the presence of sulfate and chloride ion and in the presence of Na and Ba. It has been shown by Loeb (18) that the osmotic pressure, viscosity, and swelling of a protein are very much less in the presence of bivalent ions than in the presence of monovalent ions at the same pH. It has also been shown that the amount of acid required to bring the protein solution to a given pH is independent of the nature and valence of the anion. In other words the chemical effect of all acids is the same, but the effect on the physical properties is different for various acids. It has also been shown by Loeb that this effect on the physical properties has nothing to do with the degree of hydration, but is the result of an equilibrium governed by the law of mass action, the theory of which was stated by Donnan (12). If the rate of hydrolysis, then, depends on any physical property of the protein, there should be a marked difference in the rate of hydrolysis of gelatin in the presence of sulfuric and of hydrochloric acid. The results of an experiment made to test this point are shown in Fig. 2. The figure shows the rate of hydrolysis of gelatin by pepsin in the presence of sulfuric acid and of HCl, and of gelatin and casein by trypsin in the presence of Ba and Na. As the figure shows, there is no difference whatever in the rate of hydrolysis in sulfuric acid as compared to HCl solution nor in Ba(OH)₂ as compared with NaOH (of the same pH), although the swelling, viscosity, and osmotic pressure are very much less in the presence of the divalent ions.

In the case of two solutions in one of which the protein is soluble and in the other insoluble, it would be expected that there would be a difference in the rate of digestion, no matter what mechanism is assumed to govern the reaction, since the concentration of the protein in the solution is entirely different in the two cases. In the insoluble case the protein salt is concentrated at the bottom of the vessel while in the soluble case it is distributed throughout the solution.

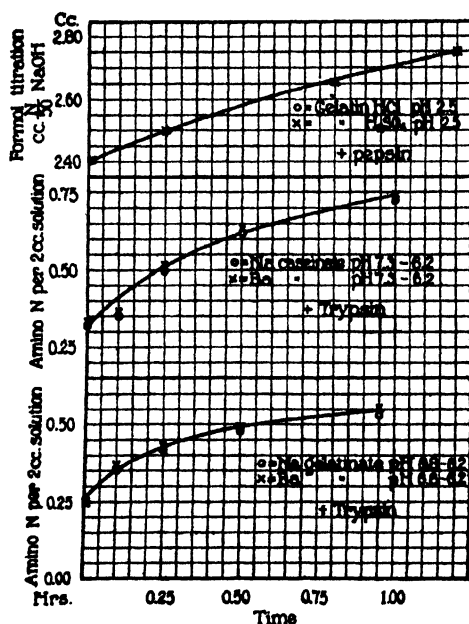


FIG. 2.

Even here, however, there is no striking difference in the rate of digestion, as shown by the writer's experiments (19) with edestin and casein in hydrochloric and sulfuric acids and Gyemant's (20) experiments with sulfosalicylic acid.

Relation between the Titration Curve and the Rate of Hydrolysis.—The experiments described above show that the effect of the acid and alkali is primarily on the chemical condition of the protein. Increasing addition of acid or alkali causes the formation of increasing amounts of protein salt. The increase in this amount of salt is shown

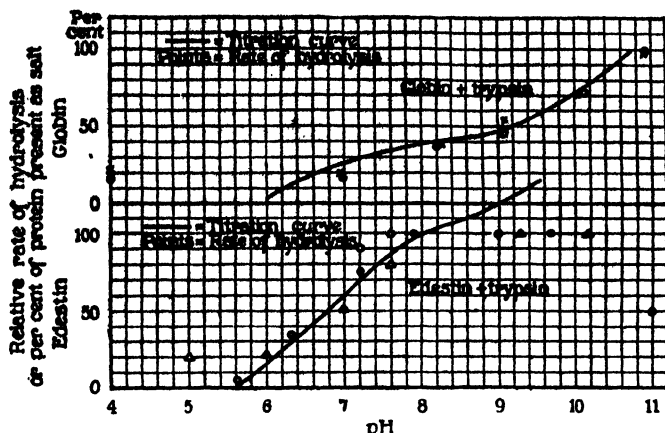


FIG. 3.

by the titration curve. If the increase in the rate of hydrolysis is due to the fact that the enzyme reacts with the ionized protein, the increase in the rate of hydrolysis with increasing acid or alkalis should be identical with the titration curve. The results of the experiments are shown in Figs. 3 and 4. The figures show that in general the

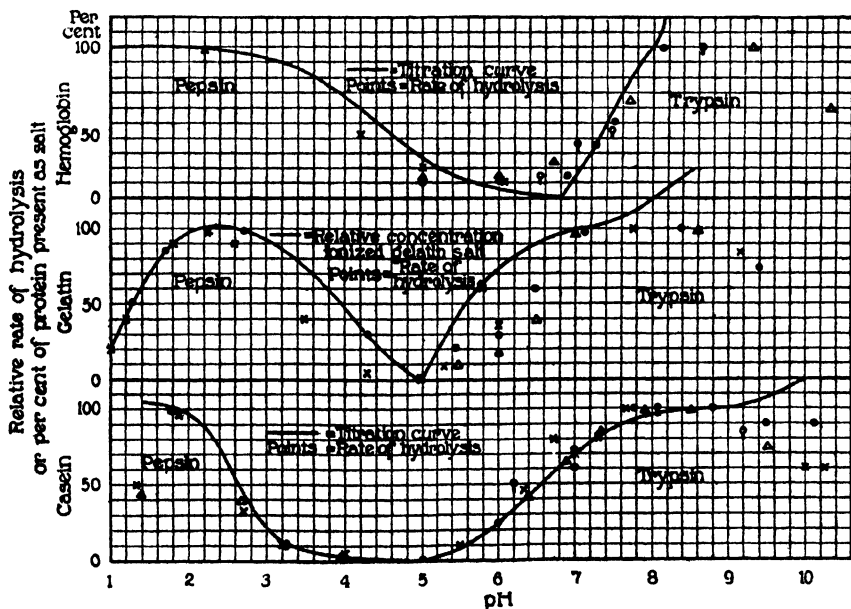


FIG. 4.

effect on the enzyme, as has been proposed by Gyemant (20) it would be expected that the descending curves would all be the same.

The experiments as a whole substantiate the idea that the reaction of the protein and enzyme depends on the concentration of ionized protein, although the experiments do not all show quantitative agreement with this mechanism. Pepsin reacts with positively charged protein ions and trypsin with negatively charged protein ions. This view is also supported by the fact that when the protein is insoluble, it combines with pepsin only when the protein is positive (21). It is also in agreement with the result of Pekelharing and Ringer (3) who found that pure pepsin is negatively charged.

A marked difference between the rate of reaction of ionized and unionized substances is the general rule in all chemical reactions and has been found to be at the basis of many reactions; it is not surprising, therefore, that it should be found to hold also in the case of protein hydrolysis. It may be remarked that the writer (10) has found that in the case of gelatin hydrolyzed by acid or alkali without any enzyme, the relative ease of hydrolysis is reversed and the results can be accounted for on the assumption that the unionized gelatin is more rapidly attacked.

An objection to this point of view which presents itself is the fact that in the case of sugar and other substances, it can hardly be supposed that the acid or alkali could affect the substrate in such a way as to give a titration curve. Michaelis and Rothstein (22) have recently suggested that in the case of sugar, a compound is formed between the invertase and the sugar, and that it is the dissociation of this compound that regulates the hydrolysis.

SUMMARY.

1. The effect of the addition of acid on the amount of ionized protein has been compared with the effect on the rate of digestion of gelatin, casein, and hemoglobin by pepsin.

2. A similar comparison has been made of the addition of alkali in the case of trypsin with gelatin, casein, hemoglobin, globin, and edestin.

3. In general, the rate of digestion may be predicted from the amount of ionized protein as determined by the titration curve or

conductivity. The rate of digestion is a minimum at the isoelectric point of the protein and a maximum at that pH at which the protein is completely combined with acid or alkali to form a salt.

4. The physical properties of the protein solution have little or no effect on the rate of digestion.

BIBLIOGRAPHY.

1. Sörensen, S. P. L., *Biochem. Z.*, 1909, **xxi**, 131.
2. Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914.
3. Pechelharng, C. A., and Ringer, W. E., *Z. Physiol. Chem.*, 1911, **lxxv**, 282.
4. Euler, H., *Z. Physiol. Chem.*, 1907, **li**, 213.
5. Arrhenius, S., *Quantitative laws in biological chemistry*, London, 1915.
6. Northrop, J. H., *J. Gen. Physiol.*, 1920-21, **iii**, 211.
7. Northrop, J. H., *J. Gen. Physiol.*, 1921-22, **iv**, 230.
8. Prepared as described by Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, **xxxv**, 147.
9. Van Slyke, D. D., *J. Biol. Chem.*, 1913-14, **xvi**, 121.
10. Northrop, J. H., *J. Gen. Physiol.*, 1920-21, **iii**, 715. The same method has been independently described by Lüters, H., *Biochem. Z.*, 1920, **civ**, 39.
11. Robertson, T. B., *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, 1918.
12. Loeb, J., *J. Gen. Physiol.*, 1921-22, **iv**, 73.
13. Northrop, J. H., *J. Gen. Physiol.*, 1921-22, **iv**, 261.
14. Ringer, W. E., *Z. Physiol. Chem.*, 1921, **cxvi**, 107.
15. Northrop, J. H., *J. Gen. Physiol.*, 1918-19, **i**, 607.
16. Ostwald, W., *Kol. Z.*, 1922, **xxx**, 243.
17. Brücke, E., *Sitzungsb. k. Akad. Wissensch. Math.-naturw. Cl.*, 1859, **xxxvii**, 131.
18. Loeb, J., *Proteins and the theory of colloidal behavior*, New York, 1922.
19. Northrop, J. H., *J. Gen. Physiol.*, 1918-19, **i**, 607.
20. Gyemant, A., *Biochem. Z.*, 1920, **cv**, 155.
21. Northrop, J. H., *J. Gen. Physiol.*, 1919-20, **ii**, 113.
22. Michaelis, J., and Rothstein, M., *Biochem. Z.*, 1920, **cx**, 217.

RESTORATION OF THE NORMAL CARDIAC MECHANISM IN AURICULAR FIBRILLATION BY QUINIDINE.

PRELIMINARY REPORT.

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Quinin has been used by physicians for its supposed sedative action on the heart for more than fifty years. In 1914, Wenckebach¹ recorded two cases of auricular fibrillation in which the normal cardiac rhythm was restored after administration of 1 gm. of this drug. The effect, however, was of short duration. In one patient, in whom fibrillation occurred in paroxysms, quinin was given repeatedly over a period of years, each time with achievement of the desired therapeutic result. Finally, no effect could be obtained and fibrillation of the auricles persisted. In a number of other patients similarly treated, no alteration in the mechanism of the heart was noted.

Stimulated by these observations, Frey,² in 1918, undertook to study the clinical pharmacology of various cinchona derivatives (quinin, quinidin and cinchonin) in patients with auricular fibrillation. He found that the most effective therapeutic agent was quinidin, which is a dextrorotatory stereo-isomer of quinin. Because of its better solubility, quinidin sulphate, rather than the pure quinidin alkaloid, was chosen for more extensive use. In his first communication Frey reported ten cases, in six of which he was able to convert the fibrillatory mechanism into the normal sinus rhythm.

During the last three years, reports of one hundred and one cases of auricular fibrillation treated with quinidin have appeared in the

1. Wenckebach, K. F. Die unregelmässige Herztätigkeit und ihre klinische Bedeutung, 1914, pp. 125 and 128.

2. Frey, W.: Ueber Vorhofflimmern beim Menschen und seine Beseitigung durch Chinidin, Berl. klin. Wchnschr. 55: 417, 451, 1918.

foreign literature;³ in fifty-nine (58.4 per cent) the normal rhythm was restored. The duration of effect has usually been short, from a few days to a month. The longest period during which normal rhythm was observed was four months. The usual dosage employed has been 0.4 gm. by mouth three times daily before meals, or, less commonly, 0.2 gm. five times a day. Regularization of the rhythm has occurred, for the most part, on the second or third day of treatment, though as much as 11 gm. has on occasion been required to produce the desired effect. It has been found advisable to give a preliminary dose of from 0.2 to 0.4 gm. to test for the possible presence of an idiosyncrasy to alkaloids of the cinchona group; for in two cases in Frey's series, grave, though not fatal, symptoms, evidenced chiefly by depression of the respiratory center, occurred after a total of 1.8 gm. had been given. Unpleasant effects, such as palpitation, headache, diarrhea, nausea or vomiting have occasionally been noted. A tachycardia usually preceded the onset of normal rhythm. Electrocardiograms made while the heart rate was rapid have in some instances shown the presence of auricular flutter as the transitional mechanism. In other cases, no transition between fibrillation and normal rhythm has been demonstrated. In still a third group, in which it was not possible to establish normal rhythm, the character of the waves in the curves showing fibrillation has temporarily become more coarse or there has been transient auricular flutter with reversion to fibrillation. Inversion of the T-wave of the electrocardiogram and prolongation of P-R (conduction) time have also been observed. There has been no significant effect on urinary output or blood pressure.

3. Frey, W.: Weitere Erfahrungen mit Chinidin bei absoluter Herzunregelmässigkeit, Berl. klin. Wchnschr. 55: 849, 1918. Von Bergmann, G.: Zur Chinidinterapie des Herzens, München. med. Wchnschr. 66: 705, 1919. Klewitz, F.: Ueber Chinidin bei Vorhofflimmern, Deutsch. med. Wchnschr. 46: 8 (Jan. 1) 1920. Schott, E.: Zur Frage der Chinidinterapie, Deutsch. Arch. f. klin. Med. 134: 208 (Feb.) 1920. Wisser: Inaug. Diss., Cologne, 1920 (cited by Schott). Leschke, E., and Ohm, R.: Die Beurteilung therapeutischer Erfolge bei Vorhofflimmern mit Herzinsuffizienz auf Grund der Ohmschen Venenpulskurve, München. med. Wchnschr. 68: 65, 1921. Benjamin and von Kapf: Ueber die Behandlung der Arrhythmia perpetua mit chinidin, Deutsch. med. Wchnschr. 47: 10, 1921. Jenny, E.: Chinidin als Herzmittel, Schweiz. med. Wchnschr. 51: 272 (March) 1921.

Knowledge concerning the mode of action of quinidin on the mammalian heart is meager. Hofmann⁴ has shown in dogs' hearts perfused with solutions of the drug a diminution in the excitability of the auricular muscle to electrical stimulation. Strong induction shocks, effective in producing fibrillation of the auricles when the heart was perfused with Locke's solution failed to elicit this arrhythmia when quinidin was added to the perfusate. The heart rate was slightly slowed. This is the only bit of experimental evidence on which a theory of the mode of action of the alkaloid may be based. In the isolated frog's heart, Santesson⁵ many years ago showed that quinidin caused retardation of rate and diminution of the force of muscular contraction.

Up to the present time, quinidin sulphate has been administered by mouth, in gelatin capsules, to four patients in the Hospital of the Rockefeller Institute. These patients have been under observation prior to treatment for periods ranging from one month to one and a half years, and are known to have been fibrillating for at least these lengths of time.

On the days on which quinidin was given, electrocardiograms were made every one or two hours and were taken several times daily thereafter. In two cases a normal sinus rhythm was restored. In one of these, auricular flutter intervened between fibrillation and the normal rhythm; in the other, an intermediary transitional mechanism, not sufficiently regular to be classed as flutter, was recorded. In the third patient, transient auricular flutter of only a few hours' duration was followed by a return to fibrillation. In the fourth case there was a temporary increase in heart rate without alteration of mechanism. In this patient complaint of headache and palpitation, together with the appearance of occasional ventricular premature contractions, induced discontinuation of the drug.

That the cardiac mechanism may be so strikingly and quickly altered by relatively small amounts of an alkaloid which exerts its

4. Hofmann, F. B.: Ueber Vorhofflimmern und seine Unterdrückung durch Chinidin, *Ztschr. f. Biol.* 71: 47, 1920.

5. Santesson, C. G.: Ueber die Wirkung einiger China-Alkaloide auf das isolierte Froschherz und auf den Blutdruck des Kaninchen, *Arch. f. exper. Path. u. Pharmakol.* 32:321, 1893.

action on the heart without producing any disturbing systemic effects is truly remarkable and worthy of note. It is for this reason that the appended cases are now recorded in this preliminary report. An extensive study of quinidin, both clinical and experimental, is in progress.

Report of cases.

Case 1 (Hospital No. 4330).—S. G., a man, aged 26, Russian, machine operator, whose case was diagnosed as chronic valvular heart disease; mitral stenosis and incompetence; auricular fibrillation, was admitted, Feb. 19, 1921, complaining of cough, dyspnea and precordial pain. There was no history of any infectious disease, nor was the patient subject to sore throats. He was first aware of having heart disease in 1913, when he suffered from palpitation and spat up a cupful of bright red blood. He had twice been a patient in other hospitals on account of his cardiac condition. He was rejected for army service in 1917 because of heart disease. During the two months prior to admission, dyspnea and cough had increased, and he had pain in the region of his heart.

Physical examination revealed moderate cyanosis of the cheeks, lips, ears and fingers. The heart was enlarged, extending 4 cm. to the right in the fourth interspace, and 16.5 cm. to the left in the sixth interspace. The signs were those of mitral stenosis and incompetence with complete cardiac irregularity. The heart rate varied from 80 to 120, with 60 to 95 beats coming through at the wrist. The blood pressure was 120 mm. of mercury systolic, 80 diastolic. There were râles over both lower lobes of the lungs. The liver was not enlarged. There was no visible edema.

With rest, fluid intake restricted to 1,500 c.c., and a light diet, the weight fell from 61.2 to 58.2 kg. in a week. Cough and dyspnea became less troublesome. The râles in the lungs disappeared. The heart rate was extremely labile, but did not significantly change its general level. He received no medication until March 17; on this day he was given quinidin sulphate, 0.2 gm., at 11 a.m. and at 1 p.m. No objective or subjective changes followed this small preliminary dose, which was given to test for the presence of a drug idiosyncrasy.

March 18, he received quinidin sulphate, 0.4 gm., at 7, 9 and 11 a.m. An electrocardiogram made at 9:35 a.m. showed that the auricles were fluttering; at 12:05 p. m. a normal sinus rhythm with rate of 94 was apparent (Fig.1). The patient was unaware of the fact that the cardiac mechanism had altered, and noted no unusual symptoms which might have been ascribed to the quinidin. It is of interest that the T-wave, which was downwardly deflected when normal rhythm was first established, became upright on the following day.

As is evident from the chart (Fig.2), the heart rate continued to fall, and for twelve days following treatment ranged from 45 to 72 per minute. There was no noteworthy change in either systolic or diastolic blood pressure. The urinary

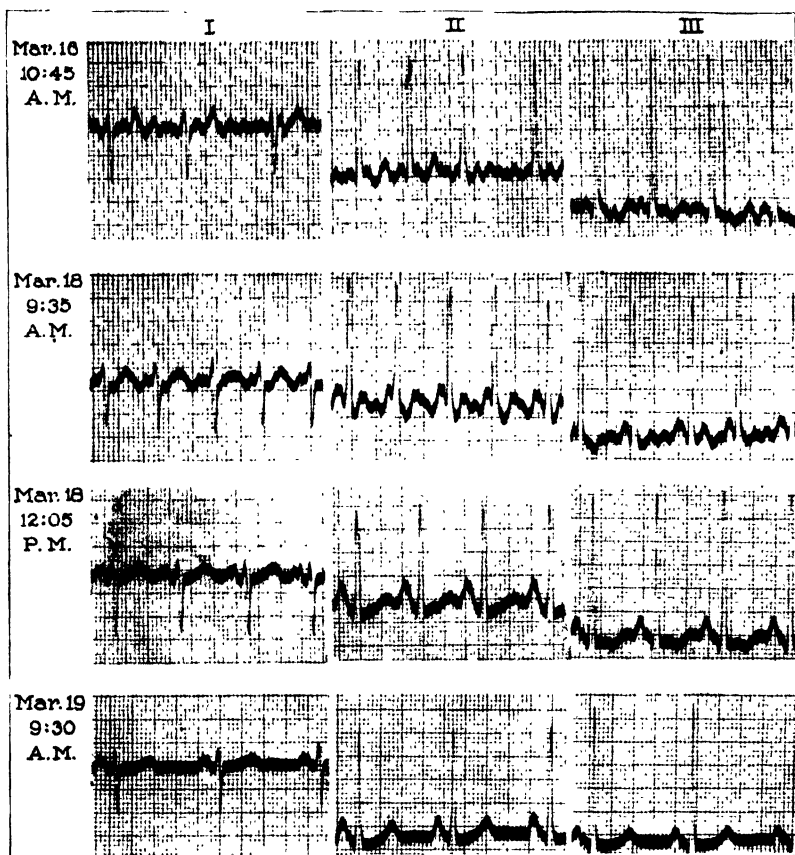


FIG. 1. (Case 1).—March 16, control, auricular fibrillation and right ventricular preponderance. March 17, quinidin sulphate, 0.4 gm. March 18, quinidin sulphate 0.4 gm. at 7, 9, and 11 a. m. Electrocardiogram at 9:35 a. m. shows auricular flutter. At 12:05 p. m., normal rhythm, with rate 94, P-R time 0.18 to 0.19 second, T-wave negative. March 19, rate 64, P-R time 0.16 to 0.17 second, T-wave positive.

output was slightly diminished on the day of quinidin administration, and somewhat exceeded the usual level during the next twenty-four hours. The total amount of quinidin given was 1.6 gm. in two days. The patient remained comfortable, and on March 23 was allowed out of bed in a chair for the first time. The normal rhythm was maintained for twelve days. On the thirteenth day, the auricles again began to fibrillate, the heart beating at the rate of 90 per minute, with pulse deficit of 8. As before, the patient was unaware of the fact that the cardiac mechanism had altered.

Case 2 (Hospital No. 3968).—P. O., a man, aged 54, boiler maker's assistant, diagnosed as having syphilis, chronic myocarditis and auricular fibrillation, was first admitted to the hospital, Sept. 29, 1919, complaining of palpitation and a sense of oppression in the chest. He had two mild attacks of rheumatism, the first in 1912, the second ten months before entrance to the hospital. He had a chancre at the age of 23, and received antisyphilitic treatment for one year. He had been a heavy drinker up to the age of 40, and was an immoderate smoker and hard worker. His present illness began six months before admission with a smothering sensation in the chest; two weeks later edema of the feet appeared, and he was put to bed for three weeks under a physician's care. Since that time he had been unable to return to work, and complained particularly of the fact that his heart "pounded like an engine," especially at night.

Physical examination disclosed very slight dyspnea, a somewhat enlarged heart, extending 4 cm. to the right in the fourth interspace, 13 cm. to the left in the fifth interspace, and total cardiac irregularity. There was no evidence of valvular disease. The blood pressure was 142 systolic and 70 diastolic. There was no edema. The Wassermann reaction on the blood serum was + + + +. The patient remained in the hospital until Jan. 20, 1920, during which time his heart rate was extremely labile, ranging from 130 to 150, with a large pulse deficit when undigitalized, but responding promptly to digitalis or strophanthin therapy. He left the hospital with instructions to continue taking digitalis each day. Because of vertigo and palpitation he was readmitted, May 27, and remained in the hospital until Aug. 27, 1920. Since that time he continued to take digitalis, 0.2 gm. each day, but did not return to work. He presented himself for examination about once a month. Throughout this entire period, during which electrocardiograms were frequently made, the cardiac mechanism remained constantly that of auricular fibrillation with occasional premature contractions of ventricular origin. He continued to take 0.2 gm. of digitan each day up to the time of the present readmission, March 10, 1921.

Physical examination at this time showed the heart to be of about the same size as on the first examination, with auricles still fibrillating. The blood pressure was 124 systolic and 78 diastolic. The heart rate was from 90 to 95, with radial rate from 85 to 90. Digitalis was discontinued, and the administration of quinidin sulphate begun.

The results of quinidin treatment were as follows (Fig. 3):

March 10, 0.2 gm. at 3 and 6 p. m. No effect was evident.

March 11, 0.2 gm. at 7, 9 and 11 a. m. and at 1 and 3 p. m. Tachycardia and palpitation followed, but were of only several hours' duration.

March 12 and March 13, the drug was omitted.

March 14, 0.4 gm. at 8 a. m., 12 noon and 4 p. m. There was an increase in heart rate to from 125 to 130, with a deficit of from 12 to 15 beats, but no discomfort.

March 15, 0.4 gm. at 8 and 10 a. m., 12 noon and 2 and 4 p. m. The heart rate continued rapid, ranging from 120 to 130, with from 80 to 90 beats coming through

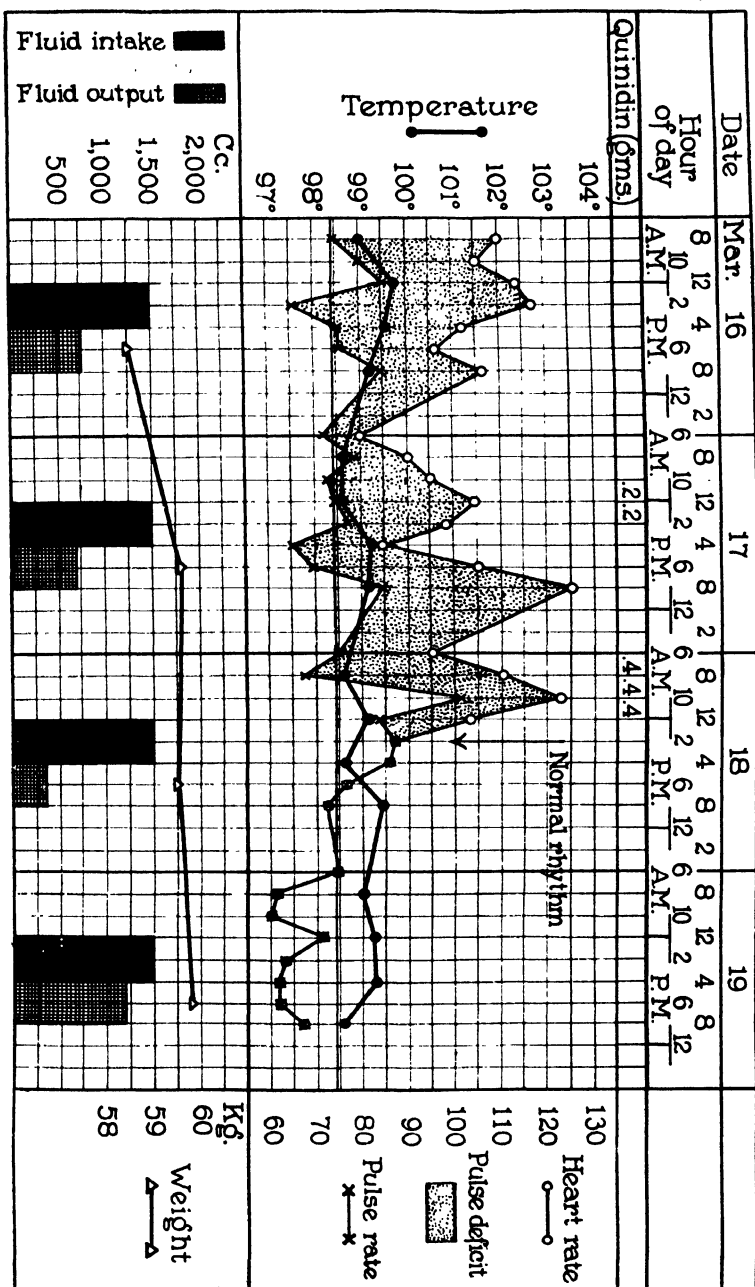


FIG. 2. (Case 1).—Clinical chart.

at the wrist. An electrocardiogram made at 2:50 p. m., after 1.6 gm. had been given on this day, showed a transition between fibrillation and normal mechanism. There may be seen a number of waves, which are to be regarded as evidence of auricular contractions rather than of fibrillation. They are not, however, of sufficient regularity to justify designating the mechanism as auricular flutter. An electrocardiogram at 4:45 p. m. revealed establishment of the normal sinus rhythm, with a rate of 80. The patient was unaware of the altered cardiac mechanism until that night, when he remarked upon the absence of the usual "pounding" of his heart. During the following twenty-four hours the heart rate gradually fell to 70, and has remained between 55 and 70 for the last fifteen days. There are occasional premature auricular contractions. It is worthy of note that the T-wave of the electrocardiogram made on March 15 is upwardly directed in Leads II and III, whereas, in the curve made the following day, the corresponding deflections are in the downward direction. There was slight increase in the urinary output on the two days following quinidin administration. There was a slight fall in systolic blood pressure on the day after quinidin was discontinued, namely, from 128 to 104 mm. of mercury. The diastolic level remained unchanged; by the following day the systolic pressure had returned to its former level. The patient is comfortable and is now up and about. The total amount of quinidin given was 4.6 gm. in six days.

Case 3 (Hospital No 4274).— M. R., a schoolboy, aged 18, American, whose case was diagnosed as chronic valvular heart disease; mitral stenosis and incompetence; aortic incompetence; auricular fibrillation; chronic tonsillitis; tonsillectomy, was admitted, Nov. 29, 1920, complaining of hemoptysis, palpitation and precordial pain. He was subject to frequent attacks of tonsillitis, and had two attacks of rheumatic fever—one at 11, the second at 12 years of age. He first noticed dyspnea on going upstairs in 1912, and was then told that he had rheumatic heart disease. He continued at school though under observation by his physician, with alternating periods of comfort and of ill health. During the month preceding admission to the hospital, dyspnea increased and he had two hemoptyses.

Physical examination disclosed large, infected tonsils. Cardiac dulness extended 5 cm. to the right in the fourth interspace and 14 cm. to the left in the sixth interspace. There were signs of both mitral and aortic valvular disease, and auricular fibrillation. The blood pressure was 126 systolic and 30 diastolic. For six weeks the boy was kept in bed without medication. The heart rate varied from 80 to 95, with pulse deficit from 10 to 15. Occasional palpitation was the only complaint. January 28, tonsillectomy was performed under local anesthesia; convalescence was uneventful, the cardiac condition remaining essentially unchanged.

Quinidin sulphate was given as follows:

February 3, 0.1 gm. at 12 noon and 5 p. m.

February 4, 0.2 gm at 12 noon and 5 p. m. There was no evident effect.

February 5, 0.2 gm. at 10 a. m., 12 noon, 4 and 8 p. m. After the third dose there was some acceleration of heart rate of which the patient was aware.

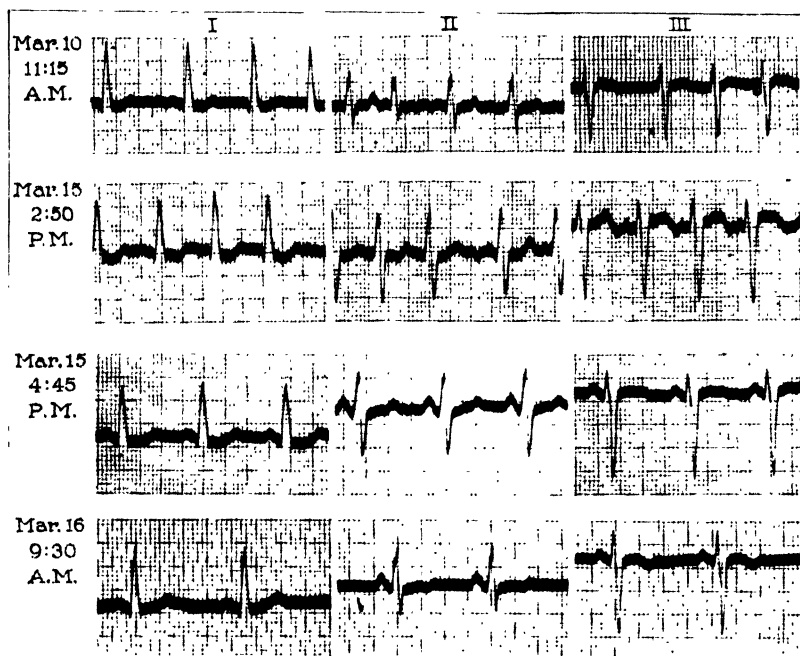


FIG. 3. (Case 2).—March 10, control, shows auricular fibrillation and left ventricular preponderance. Quinidin sulphate, 0.4 gm. March 11, quinidin sulphate, 1 gm. March 12 and 13, drug omitted. March 14, 1.2 gm. March 15, quinidin sulphate, 0.4 gm. at 10 a. m., 12 noon, and 2 and 4 p. m. Electrocardiogram at 2:50 p. m., after 1.6 gm. had been given on this day, shows a transition between fibrillation and the normal mechanism. There are seen a number of waves which are to be regarded as evidence of auricular contractions rather than as fibrillation. They are not, however, of sufficient regularity to justify designating the mechanism as auricular flutter. Electrocardiogram at 4:45 p. m. shows a normal sinus rhythm, with rate of 80, P-R time 0.15 to 0.16 second, T-wave positive. March 16, rate 70, P-R time 0.15 to 0.16 second, T-wave negative. Occasional auricular premature beats, not shown in section of curve reproduced.

February 6, the drug was omitted.

February 7, 0.4 gm. at 10 a. m. and 4 p. m. The patient complained of palpitation and headache. The heart rate at 8 p. m. rose to 126. The electrocardiogram revealed a number of left ventricular premature beats.

February 8, 0.4 gm. at 10 a. m. and 1 and 5 p. m. The heart rate was more rapid. An electrocardiogram at 9:35 a. m. revealed a series of fibrillatory waves

coarser than on the previous day, and more ventricular extrasystoles. At 5:55 p. m. the electrocardiogram showed in places a mechanism resembling auricular flutter, in other portions coarse auricular fibrillation. The ventricular premature contractions were quite numerous. During the night the tachycardia subsided.

February 9, no ventricular extrasystoles were seen; 0.4 gm at 11:30 a. m., and 1:30 and 3:30 p. m. The heart rate again became quite rapid, but the extrasystolic arrhythmia did not reappear.

February 10, there was marked tachycardia, the rate rising to 130, with a pulse deficit of 25. The temperature likewise was elevated in the evening, reaching 102°. At this time the patient complained of precordial pain. Physical signs were unchanged. A total of 4.6 gm. of quinidin sulphate was given during a period of seven days. The following day the electrocardiogram revealed a return to fine fibrillation. The temperature was normal, and the heart rate had assumed its usual level. The urinary output during this entire period remained essentially unchanged. The further course of the patient in the hospital was not remarkable.

*Case 4 (Hospital No. 3983).—*R. R., a woman, aged 31, Russian, housewife, with a diagnosis of chronic valvular heart disease; mitral stenosis and incompetence; aortic incompetence; auricular fibrillation, was first admitted to the hospital, Oct. 23, 1919, complaining of shortness of breath and weakness. There was no history of the usual infectious diseases. She was told at the age of 18 that she had heart disease, but had no symptoms save occasional dyspnea after strenuous exertion. The birth of two children and several abortions put an unusual strain on her heart, and for the last two years there was progressively increasing dyspnea.

Physical examination disclosed moderate cardiac enlargement, dullness extending 4 cm. to the right in the fourth interspace, and 12 cm. to the left in the fifth interspace. The signs were those of mitral and aortic valvular disease with auricular fibrillation. There were râles over both lower lobes of the lungs, but there was no edema of the extremities. The heart rate was rapid, from 100 to 130, with a pulse deficit of from 20 to 40. On this, as on two subsequent admissions to the hospital, there was prompt response to digitalis or strophanthin therapy. The patient was kept under continuous observation, reporting for examination about once a month.

The present admission, Feb. 14, 1921, was because of pain in the left side, believed to be due to a small infarct in the left kidney. February 19, she received digitalin, 0.6 gm. The symptoms and signs promptly abated.

Quinidin sulphate was given as follows:

February 24, 0.2 gm. at 12 noon and 5 p. m. She complained of headache in the evening.

February 25, 0.4 gm. at 1 and 6 p. m. At 8 p. m. there was a sharp rise in heart rate with complaint of headache.

February 26, the heart rate resumed its usual level; the headache was gone. On this day, as well as on February 27 and 28, she received 0.4 gm. at 10 a. m., and 2 and 6 p. m. Each dose was followed by tachycardia, which subsided within from two to four hours after the drug had been given. While the rate was rapid,

the patient complained of headache and palpitation. She received a total of 4.8 gm. in five days. Save for the appearance of an occasional right ventricular premature contraction, there was no essential change in the cardiac mechanism or in the form of the electrocardiogram.

COMMENT.

It is evident from a survey of the literature, as well as from observation of this small group of cases, that the factors which determine the success or failure of quinidin in altering the mechanism of the heart are not clear. We have been led to give the individual doses at more frequent intervals than has been recommended (Cases 1 and 2) for two reasons: first, because it was noted that in the two unsuccessfully treated patients, the effects induced by a given amount of the drug, as evidenced by tachycardia and changes in the electrocardiogram, tended to disappear in the course of from two to four hours; second, because Wiechmann⁶ has shown that the greater the fractionation of dosage, the greater is the amount of quinidin excreted. To achieve optimal therapeutic effects it seems reasonable, at least for the present, to administer as much of the drug in as short a period of time as is consistent with the factor of safety of toxicity. It is hoped that work which is now in progress may help to answer these as well as a number of related questions.

6. Wiechmann, E.: Ueber die Ausscheidung der Chinidins im Harn, *Ztschr. f. d. ges. exper. Med.* 7: 155, 1918.

CLINICAL STUDIES OF QUINIDINE.

II. ALTERATIONS IN THE CARDIAC MECHANISM AFTER ADMINISTRATION OF QUINIDINE TO PATIENTS WITH AURICULAR FIBRILLATION WITH CONSIDERATION OF CERTAIN TOXIC EFFECTS OF THE DRUG.*

By ROBERT L. LEVY, M.D.

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During the past four years, sufficient evidence has been accumulated to indicate that in a certain number of patients suffering from fibrillation of the auricles (about 50 per cent.), oral administration of quinidin sulphate serves to restore the normal cardiac rhythm. The circumstances which determine the issue are not yet altogether clear. A summary of the earlier literature, together with a preliminary report dealing with the treatment of four cases in the Hospital of the Rockefeller Institute, has already appeared.¹

The results of experiments done in this Hospital to determine the pharmacologic action of this drug have likewise been reported.² It is the purpose of the present communication to record in detail the alterations in the mechanism of the heart's action which have been observed in the first eleven patients to whom quinidin has been administered.³ A more detailed report of the experimental studies will

* Presented in abstract before the New York Academy of Medicine, Nov. 17, 1921.

¹ Levy, R. L.: Restoration of the Normal Cardiac Mechanism in Auricular Fibrillation by Quinidin; Preliminary Report, J. A. M. A. 76: 1289 (May 7) 1921.

² Cohn, A. E., and Levy, R. L.: Experimental Studies of the Pharmacology of Quinidin, Proc. Soc. Exper. Biol. & M. 18: 283, 1921. Experiments with Quinidin on Conduction and on the Refractory Period in the Dog's Heart, Proc. Soc. Exper. Biol. & M. 19: 174, 1922.

³ A preliminary account of this study was published in the Proc. Soc. Exper. Biol. & M. 19: 88, 1921.

appear shortly. A discussion of the clinical aspects of quinidin therapy is reserved for a subsequent paper in which a larger material will be considered.⁴

Material and Method.

Five hundred and seven electrocardiograms, taken of eleven patients, have been measured and analyzed. The cases represent an unselected material. They have been under observation prior to treatment for periods ranging from one month to one and a half years, and are known to have been fibrillating for at least these periods of time. After a preliminary dose of from 0.2 to 0.4 gm. quinidin to test for the presence of an idiosyncrasy to members of the cinchona group, 0.4 gm. of the drug has been given by mouth, in gelatin capsules, either three times daily or every two hours, until the establishment of normal rhythm or the appearance of untoward symptoms indicated cessation of therapy. No more than 2 gm. quinidin was administered in twenty-four hours, though treatment has been continued daily for as long as ten days.

Electrocardiograms were taken in some instances as often as every five minutes during the time when a change of rhythm was anticipated. Usually curves were made at one or two hour intervals on the days on which the drug was given and at least once daily throughout the entire period of observation. The usual three limb leads were employed. Records made in this fashion do not always permit of accurate measurement of auricular rate when the auricles are fibrillating. References to rate, therefore, unless otherwise specified, are to be taken as indicating the number of ventricular beats per minute.

Results.

The results of this electrocardiographic study are of interest in illustrating the great variety of alterations in the mechanism of the heart's action which may be induced by quinidin. They have, furthermore, a significance for clinical medicine in so far as they indicate the

⁴In this paper will be considered the symptoms attendant on administration of the drug, the duration of its action, the effects of altered rhythm on vital capacity and heart size as determined teleroentgenographically, the relationship between digitalis and quinidin therapy, etc.

manner in which the transition from fibrillation to normal rhythm may be expected to occur. But of paramount importance is the significance attached to various toxic effects which have been observed, as evidenced by the occurrence of rhythms usually associated with poisoning of the heart muscle. It is particularly desirable that those concerned with the care and treatment of patients with heart disease should be familiar with the signs and dangers of quinidin intoxication.

For the purpose of discussion, the eleven cases naturally fall into two groups: (1) three patients, in whom, on different occasions, the normal mechanism was restored nine times, and who, in all, received twelve courses of quinidin; (2) eight patients, in whom restoration of the normal mechanism was not accomplished and who received, in all, eleven courses of quinidin. Detailed case records are appended.

Cases in Which the Normal Rhythm Was Restored.

Sequence of Events.—In order to learn in what sequence the various changes in cardiac mechanism occurred, detailed analyses of all electrocardiograms were chronologically charted. A summary of the findings is given in Table I.

The first effect noted was usually acceleration of ventricular rate. On the other hand when the rate was initially very rapid (Case 1, Courses 2, 3 and 4), the drug caused a reduction in the number of ventricular contractions. Alterations in rate occurred in from one to three hours after beginning treatment. Tachycardia was followed at times by the appearance of premature beats, arising more commonly in the right, but occasionally in the left ventricle. The ectopic ventricular contractions were frequently transitory (Cases 1 and 2), but in one case (Case 3), they were the precursors of paroxysms of ventricular tachycardia, to which particular reference will be made later. If electrocardiograms were taken at sufficiently frequent intervals, the transitional mechanisms in the common order of their appearance were: coarser fibrillation, that is to say, waves in the electrocardiogram having a higher voltage and denoting retardation of auricular rate;⁵

⁵ Drury, A. N., and Iliescu, C. C.: The Restoration of the Normal Cardiac Mechanism in Cases of Auricular Fibrillation by Means of Quinidin Sulphate, *Brit. M. J.* 7: 511 (Oct. 1) 1921. These authors, by use of direct chest leads, have secured records in which accurate counts of auricular rate were possible and have noted progressive retardation after quinidin.

impure auricular flutter;⁶ auricular flutter, and finally, normal rhythm (Fig. 4). This sequence was not invariably exhibited in all its phases and it is possible that one or more of the intermediate mechanisms may be omitted.

Transition.—In one patient the transition from auricular flutter to the normal rhythm was photographed in the second lead (Fig. 5). The abruptness of the change is remarkable. The first twelve interauricular intervals in the curve range from 0.2180 to 0.2580 second (Table II) with maximum variation of 0.04 second. The corresponding phases of successive cycles are strikingly uniform in contour. The maximum variation in interventricular intervals is 0.0240 second.⁷ There follows a period of altering auricular activity, denoted by the next seven P waves, which exhibit increasingly greater deviations from the usual form. The interauricular intervals are, on the whole, longer than in the group immediately preceding, and range from 0.2280 to 0.2860 second, with maximum variation of 0.0580 second. There is coincidentally gradual widening of the interventricular periods. Then comes a relatively long asystole of both auricles and ventricles and this is followed by prompt resumption of the sinus rhythm.

A consideration of the manner in which the transition occurs is of some interest in relation to the theory that a circus excitation is

⁶ By impure auricular flutter is meant a mechanism in which auricular (P) waves are present, but in which they are somewhat irregularly spaced and of varying contour, in contradistinction to the regularity of interauricular intervals and constancy of form of corresponding P waves in pure flutter. It is to be regarded as intermediate between auricular fibrillation and flutter.

⁷ Lewis, T., in speaking of the lengths of auricular cycles in auricular flutter, states that "the maximal variation of the cycles in the same curves is usually less, probably considerably less than 0.01 second; probably it may amount to, but does not materially exceed, 0.02 second on occasion. It seems advisable that the term auricular flutter should be confined to disorders, be they clinical or experimental, in which comparable variations in the lengths of auricular cycles are found not to be exceeded." Heart 7: 127, 1920. These statements are based on measurements of the curves of seven cases of flutter, in which interventricular, not interauricular intervals, were determined. In one of these cases the maximal variation in interventricular intervals was 0.0265 second. A discussion of the validity of Lewis' criteria is not within the scope of this paper. There seems no reason to doubt that the curve shown in Figure 5 is a record of auricular flutter.

TABLE I.
Summary of Cases in Which the Normal Mechanism Was Restored.

Case and Ref. No.	Clinical diagnosis	Quinidin, gm.	Duration of attack, days	Original mechanism	Effect on			Mechanism after quinidin										Remarks		
					Rate	P-R sec.	T	QRS	Coarse fb.	Impure flutter.	Fib. + imp. flutter.	Flutter.	Normal rhythm.	VPCB	VFL.	APC	Vent tachy		S-A block.	Aur. tachy.
1. 3968	Syphilis; chronic myo- carditis.	4.6	6	Aur. fib.,* VPL, VPCB	+ (1)†	0.13-0.15	+ (5)			+ (3)			+ (4)	+ (2)		+ (6)			Patient partly digitalized prior to quinidin administration; APC appeared after onset of normal rhythm.	
		1.2	1	Aur. fib.; VPL	- (2)	0.12-0.14	+ (3)	+ (5)		+ (4)		+ (6)	+ (7)	+ (1)		+ (8)			Patient not digitalized; ven- tricular rate 185 before quinidin administration; APC again appeared after onset of normal rhythm; 6 days after resumption of QRS was noted, it re- turned to normal duration.	
		0.4 0.4 1.2	1 1 1	Aur. fib.; VPL Aur. fib.; VPL Normal rhythm; VPL APC, VPCB	- (1) - (2)	0.12-0.13 0.12-0.14	+ (3) + (4)		 + (1)	 + (2)	 + (3)	 + (4)	 + (5)	 + (6)	 + (7)	 + (8)	 + (9)	 + (10)	APC and VPCB disappeared after quinidin. T wave changed and APC ap- peared after onset of normal rhythm; impure flutter inter- vened between normal rhythm and reversion to fibrillation. APC incessant.	
2. 4330	Chronic endocarditis; mi- tral stenosis and insuffi- ciency.	1.6	2	Aur. fib.; VPCB	+ (1)	0.16	+ (5)					+ (2)	+ (4)		+ (3)	+ (6)				
		0.8 0.4 1.2	1 1 1	Aur. fib.; VPCB Aur. fib.; impure flutter, flutter; Aur. fib.	- (1) - (3)	0.14-0.16 0.16-0.17	+ (3) + (5)		 + (1)	 + (2)	 + (2)	 + (2)	 + (4)	 + (5)	 + (6)	 + (7)	 + (8)	 + (9)	 + (10)	T wave change transient; sino- auricular block observed once after onset of normal rhythm; one paroxysm of auricular tachycardia after onset of nor- mal rhythm. Normal rhythm maintained for less than 24 hours.
3. 4118	Chronic endocarditis; mi- tral stenosis and insuffi- ciency.	8.4	7	Aur. fib.	+ (1)	0.21-0.24	+ (8)			+ (6)	+ (4)		+ (7)	+ (2)	+ (5)	+ (11)	+ (3)	+ (9)		
		6.4	5	Aur. fib.	- (2)	0.21	+ (7)			+ (5)	+ (3)		+ (6)	+ (1)	+ (4)					
		1.6	1	Aur. fib.	+ (1)						+ (2)			+ (4)	+ (5)		+ (3)			

* Key to observations used in Tables I and II:

Aur. fib.: Auricular fibrillation.

VPCB: Ventricular premature contractions, right.

VPL: Ventricular preponderance, left.

VPCB: Ventricular premature contractions, left.

† The numbers in the various columns denote the chronologic sequence of the changes observed. Under rate, + = acceleration; - = retardation.

VPCB: Ventricular preponderance, right.

VPL: Ventricular preponderance, left.

VPCB: Ventricular premature contractions, right.

VPL: Ventricular preponderance, left.

† The numbers in the various columns denote the chronologic sequence of the changes observed. Under rate, + = acceleration; - = retardation.

Vent. tachy.: Ventricular tachycardia.

Aur. tachy.: Auricular tachycardia.

S-A block: Sino-auricular block.

Vent. tachy.: Ventricular tachycardia.

Aur. tachy.: Auricular tachycardia.

S-A block: Sino-auricular block.

the nature of the disturbed mechanism at the basis of fibrillation and flutter of the auricles. According to this concept, first clearly defined by A. G. Mayer, working with the umbrella of the jelly fish, developed and demonstrated in heart muscle by Garrey and by Mines, and more recently elaborated and applied to human physiology by Lewis, cessa-

TABLE II.

*Measurement of Electrocardiogram (Lead II) Showing Transition from Auricular Flutter to Normal Rhythm.**

Cardiac mechanism.	Interauricular intervals, second.	Interventricular intervals, second.	P-R time, second.
Auricular flutter.....	0.2324		
	0.2510	0.4520	
	0.2260		
	0.2360	0.4760	
	0.2180		
	0.2288	0.4608	
	0.2200		
	0.2230	0.4606	
	0.2212		
	0.2210	0.4530	
	0.2580		
	0.2210	0.4558	
Altering auricular activity.....	0.2455		
	0.2362	0.4936	
	0.2280		
	0.2860	0.5760	
	0.2610		
	0.2420	0.6310	
Transition.....	1.0889	1.0349	
Normal rhythm.....	0.7720	0.7752	0.1696
	0.8200	0.8256	0.1632
	0.8450	0.8456	0.1640
	0.8594	0.8610	0.1620

* Measurements were made with the use of a projection apparatus.

tion of auricular fibrillation or flutter may, on theoretic grounds, be induced in three ways: (1) by lengthening the path of the excitation wave in the auricle; (2) by accelerating the rate of conduction of the impulse through the auricular muscle; (3) by lengthening the refractory period of this muscle. It is clear from the electrocardiogram

shown in Figure 5, that immediately before and during the transition the excitation wave is traversing a different path from that travelled during the preceding period of flutter; for the P waves alter in form. They are, moreover, irregularly and more widely spaced. To which of the three conceivable causes just mentioned this change may be ascribed, it is not possible definitely to establish. It is known, however, that in the dog's heart, quinidin retards the rate of conduction of the impulse through the muscle of the auricle and prolongs the refractory period.²⁻³ It furthermore raises the threshold of excitability of the auricle, so that a faradic current adequate to induce fibrillation prior to the injection of quinidin does not suffice to cause the arrhythmia after the drug has been given.² It is probable that the resultant of these effects determines whether cessation of fibrillation takes place. Slowing of the conduction rate may be regarded as exerting an unfavorable or negative effect in arresting the circus movement, whereas the raised threshold of excitability and more particularly, lengthening of the refractory period, exercise a favorable or positive effect in promoting this result. The decreased rate of the flutter waves in this instance at all events indicates that probably a longer path, perhaps at a slower rate, was traversed by the excitation wave. The alteration in path may have been necessitated by an increase in the refractory period.

After the resumption of normal rhythm, the auricular waves, though distinct, showed at times decided variations both in contour and voltage. Evidence of such change in the P waves (Fig. 5) in Cycles 14 and 39 is striking. There is apparently still a tendency for the impulse to be initiated at a point other than at the sinus node or to traverse an unusual and sinuous path in its course through the auricle.

Ectopic Beats; Auricular Tachycardia; Sino-Auricular Block.—Ectopic auricular contractions were observed in all three cases at times after the normal mechanism was resumed. Their occurrence points to the persistent presence of a point of origin away from the sinus node, and probably within the musculature of the auricle. Additional evidence strengthening the belief that there is disturbance in both

³ Lewis, T., Drury, A. N., Iliescu, C. C. and Wedd, A. M.: Observations Relating to the Action of Quinidin on the Dog's Heart, with Special Reference to Its Action on Clinical Fibrillation of the Auricles, *Heart* 9: 55, 1921.

initiation and propagation of the impulse through the auricle in individuals suffering from atrial fibrillation is afforded by Case 3, in which, during the brief period in which normal rhythm was maintained, sino-auricular block and paroxysms of ectopic auricular tachycardia occurred (Fig. 6).

When auricular premature beats became numerous, these, as well as ectopic ventricular contractions, could be abolished by the further administration of quinidin (Case 1, fifth course; Fig. 3). It is to be noted that the ventricular premature beats disappeared earlier than those arising in the auricle.⁹

Rate.—On the day on which normal rhythm was established, the ventricular rate usually ranged from 80 to 90 and on the following day from 60 to 70, subsequently remaining at or about this level during the period in which the sequential rhythm prevailed. When the transition from the normal rhythm to fibrillation or flutter took place, there was correspondingly a gradual acceleration of rate, a level eventually being reached in the course of twenty-four hours at which the ventricles then continued to beat with relatively slight variations.

A-V Conduction.—Conduction through the auriculoventricular bundle when the sinus rhythm was present was within normal limits in two cases, as indicated by the P-R time. In the third case the P-R interval was prolonged (0.24 sec.); but it is not possible to say whether this was a drug effect or was due to interference with the passage of the impulse from other causes.

Intra-Ventricular Conduction.—In one individual (Case 1, second course), there was definite evidence that quinidin caused interference with the spread of the excitation wave through the ventricles (partial intraventricular block) (Fig. 1). The QRS interval rose from 0.10 sec. before quinidin administration to 0.13 sec. after the drug had been given, and did not return to its former value for five days. There was no coincident prolongation of A-V conduction.

⁹ We have since had occasion to test the effect of quinidin in patients showing no disturbance in rhythm other than ectopic beats arising either in auricles or ventricles. In certain of these individuals, the undisturbed normal mechanism can be restored and, if desired, maintained by proper quinidin dosage.

Alteration in Ventricular Complex.—In the series of curves just described (Fig. 1) is seen an example of change in the contour and voltage of the ventricular complex after quinidin administration. This is exhibited particularly in the R and S waves and in the altered form and position of the notching. Similar changes were observed in one other case. There is, presumably, some change in the path of the impulse through the ventricles.

T Wave.—The T wave was distinctly affected by quinidin, in that it tended to reverse its direction and change its form after the normal rhythm was established.¹ Though slight variations in this portion of the electrocardiogram were among the early effects of quinidin medication, the more striking changes occurred after the sinus rhythm had prevailed for twenty-four hours and were best seen in Lead III (Fig. 2). When the fibrillatory mechanism was resumed, the T wave usually returned to its original direction and form either at once or after a short interval. It is of interest to note that in one instance, however (Case 2, first course), the T wave change preceded the alteration in cardiac mechanism. On the last day of normal rhythm the T wave in Lead III changed from upright to inverted. On the following morning, auricular fibrillation was again recorded, the T wave remaining as on the previous day. It is, therefore, clear that, although change in the form of the T wave and change in rhythm often occur synchronously, they are not necessarily interdependent. In dogs in which quinidin was injected similar alterations in the T wave were noted.² These observations are recorded without any attempt at interpretation, since the significance of change in this portion of the electrocardiogram is by no means clear.

Influence of Digitalis.—Digitalization prior to quinidinization was not essential for success in restoring the normal mechanism. For in the same individual (Case 1), the normal rhythm was on one occasion restored after digitalis administration, with ventricular rate ranging from 90 to 100 (first course), and at another time, after no digitalis was given, when the ventricles were beating at the rate of 180 per minute (second course). A number of observations bearing on this point have been made and will be discussed more fully at another time.

Effect of Atropin.—Intravenous injection of atropin sulphate (from 1.0 to 1.5 mgm.) in these patients at a time when fibrillation was

TABLE III.
Summary of Cases in Which Restoration of the Normal Rhythm Was Not Accomplished.

Case and loop No.	Clinical diagnosis.	Quinidin, gm.	Duration of atrial fibril- lating, days.	Original mechanism.	Effect on				Mechanism after quinidin.								Remarks.		
					Rate.	P-R	T	QRS	Coarse fib.	Impure flutter.	Fib. + imp. flutter.	Flutter.	Normal rhythm.	VPCR	VPCL	APC		Ven- tucy.	
4 3983	Chronic endocarditis; mitral stenosis and insufficiency; aortic insufficiency.	4.8	5	Aur. fib.; VPR	+ (1)														
5 4274	Chronic endocarditis; mitral stenosis and insufficiency; aortic insufficiency.	4.2	7	Aur. fib.; VPR; VPCL	+ (1)					+ (4)		+ (5)	+ (6)			+ (2)	+ (3)		
6 4127	Chronic endocarditis; mitral stenosis and insufficiency.	3.6	5	Aur. fib.; VPR; VPCR (occasional)	+ (1)											+ (2)	+ (4)		Patient digitalized before beginning quinidin therapy; VPCR increased in number, at times resulting in coupled rhythm.
7 4327	Chronic myocarditis.	3.2	5	Aur. fib.; VPL	+ (3)							+ (6)	+ (5)			+ (1)	+ (3)		After digitalis, fibrillatory mechanism returned.
8 4345	Chronic endocarditis; mitral stenosis and insufficiency.	6.2	8	Aur. fib.	+ (1)		+ (3)		+ (6)	+ (4)						+ (5)	+ (2)		
9 4308	Chronic endocarditis; mitral stenosis and insufficiency.	2.4	3	Aur. fib.	+ (1)		+ (3)		+ (4)	+ (5)	+ (6)						+ (2)		
10 4349	Chronic endocarditis; mitral stenosis and insufficiency; aortic insufficiency.	3.0	4	Aur. fib.	+ (1)		+ (3)									+ (2)			Patient digitalized prior to quinidin admin- istration.
		1.6	3	Aur. fib.	+ (3)		+ (4)		+ (1)							+ (2)			Patient again digitalized before beginning quinidin.
		4.0	5	Aur. fib.	+ (1)		+ (2)												Quinidin and digitalis combined.
11 4267	Chronic myocarditis.	8.8	10	Aur. fib.; VPL	+ (2)				+ (3)								+ (1)		Patient digitalized before beginning quinidin therapy.

present and again when the normal rhythm prevailed, resulted in the usual increase in ventricular rate, but in no noteworthy alteration in the cardiac mechanism or in the form of the electrocardiogram.

Cases in Which Restoration of the Normal Mechanism Was Not Accomplished.

In this group of eight patients, evidences of the undesirable action of quinidin were more frequently observed (Table III).

Tachycardia.—As in the cases in which it was possible to establish normal rhythm, tachycardia was commonly the first effect noted and was often sufficiently troublesome to cause complaint. Having once been initiated, it was rarely prolonged beyond four or five hours after the drug was discontinued. In one patient (Case 10, first course), in whom simple tachycardia was induced by quinidin, definite signs and symptoms of heart failure ensued. There was palpitation, dyspnea, epigastric distress, large tender liver, accumulation of invisible edema, as evidenced by gain in weight and oliguria and a striking rise in venous pressure from 10.8 to 20.2 cm. of water.¹⁰ A graphic account of these findings is given in Figure 9. A course of digitalis therapy was followed by prompt disappearance of symptoms and signs of decompensation.

Coarse Fibrillation; Impure Flutter; Flutter.—Coarsening of fibrillatory waves, indicating retardation of auricular rate, was noted on five occasions. It was at times among the earliest detected changes in the electrocardiogram, but in other instances was seen as a later effect. The mechanism by which slowing is produced is not precisely to be ascertained. It is known, however, that one of the pharmacologic actions of quinidin is to depress conductivity in the auricle, and it is conceivable, and altogether likely, that slowed auricular rate is an exhibition of this effect. When still greater depression of conduction has been produced, impure flutter occurs. This was observed on three occasions in combination with periods of fibrillation and twice as the dominant rhythm. In two patients (Cases 5 and 7) impure flutter was followed by flutter, but larger dosage was not effective in restoring

¹⁰ Observations of venous pressure were made by the method of Hooker: *Am. J. Physiol.* 35: 73, 1914.

the normal rhythm. In Case 5, the flutter was of short duration, lasting but twenty minutes, after which a mixed rhythm with periods of fibrillation and impure flutter was recorded. In Case 7, however, flutter persisted for three days and was followed, after administration of digitalis, by reversion to the fibrillatory rhythm.

These observations serve to demonstrate the close interrelationship between fibrillation, impure flutter and flutter, and lend support to the concept that they are all dependent upon the same basic disturbance, namely, a circus contraction in the auricle. In these respects, Lewis' experimental data¹¹ are confirmed for the clinic.

Ventricular Premature Beats.—Of the greatest significance to the clinician are those arrhythmias which indicate irritation of the ventricular muscle. Reference has already been made, in discussing the first group of cases, to the occurrence of ectopic ventricular premature beats, which arose more often in the right, but occasionally in the left ventricle. These were observed in all of the eight cases in the series now under discussion. In some instances they were transitory, disappearing spontaneously or after more of the drug was given. They assume particular importance in those individuals in whom occasional ventricular premature beats are not present prior to the beginning of treatment. They may occur as isolated extrasystoles; or, in such fashion as to cause coupled rhythm, like that seen in the early stages of digitalis poisoning (Case 6, first course; Fig. 7).

Paroxysms of Ventricular Tachycardia.—If more quinidin is given, however, ventricular premature beats may occur in rapid succession in a manner to result in paroxysms of ectopic ventricular tachycardia (Cases 3, 6, 7 and 11, Figs. 6 and 8), or as a continuous sequence of extrasystoles arising in an ectopic focus in the ventricle (Case 7, Fig. 8). Case 7 is of particular interest in this connection, in that ventricular tachycardia occurred at first during the fibrillatory period, then at a time when auricular flutter with 3:1 block was present and subsequently, with slower auricular rate and 2:1 block. The paroxysms in the three cases in which this mechanism was seen were fortunately of short duration, lasting always less than twenty hours

¹¹ Lewis, T.: Observations upon Flutter and Fibrillation. Part IV. Impure Flutter; Theory of Circus Movement, Heart 7: 293, 1920.

and sometimes disappearing in the course of three hours after the last dose of quinidin was given.

Ventricular tachycardia assumes a real significance if it is borne in mind that in dogs injected with digitalis or strophanthin this irregularity is not infrequently the precursor of ventricular fibrillation and death. Dogs which have received quinidin occasionally, though rarely, die with this arrhythmia.² Several sudden and unexplained deaths are reported by careful observers following quinidin administration in patients in whom, at necropsy, dilatation of the heart but no evidence of pulmonary or other embolism was found. It is quite possible that in these patients ventricular fibrillation was the cause of death. Unfortunately, the cardiac mechanism shortly before and at the time of death has not been recorded.

In certain individuals, then, quinidin is a highly toxic substance for heart muscle, even in doses ordinarily regarded as safe in clinical therapeutics. Until further details of its action are known, it is wise to insist that patients receiving this drug should remain in bed, preferably in a hospital, under close observation by nurse and physician. Where possible, therapy should be controlled by graphic records. Physicians who prescribe quinidin should do so with the realization that in proper hands it possesses great therapeutic virtue, whereas unintelligently given, it may cause disastrous effects and fall into undeserved disrepute.

SUMMARY.

1. An electrocardiographic study has been made of eleven patients with auricular fibrillation who received quinidin sulphate by mouth. In three, to whom quinidin was given on twelve occasions, the normal rhythm was restored nine times. In eight, to whom the drug was given on eleven occasions, restoration of the normal rhythm was not accomplished.

2. In the cases in which normal rhythm was established, the first effect noted was usually acceleration of ventricular rate; next in order came ectopic ventricular beats. The transitional mechanisms in the common order of appearance were: coarser fibrillation, denoting retardation of auricular rate, impure flutter, flutter and normal rhythm.

3. The transition from flutter to normal rhythm has been photographed in the second lead and has been described.

4. The occurrence of variations in the form of P waves, ectopic auricular contractions, auricular tachycardia and sino-auricular block after resumption of the sinus rhythm, is adduced as evidence that there is, even at this time, a tendency for the impulse to be initiated at a point other than at the sinus node and to traverse an unusual path in its course through the auricle.

5. Both auricular and ventricular premature beats could, on occasion, be abolished by further exhibition of the drug.

6. The effects on rate, A-V conduction, intraventricular conduction and on the T wave have been discussed.

7. Reference has been made to the influence of digitalis on the issue of quinidin administration and to the effect of atropin after establishment of the sinus rhythm.

8. In the cases in which restoration of the normal mechanism was not accomplished, evidences of the undesirable action of quinidin were more frequently observed. Tachycardia was troublesome and in one instance was followed by signs and symptoms of heart failure.

9. The close interrelationship of fibrillation, impure flutter and flutter was evident in that rapid change from one of these mechanisms to the other was frequently seen. This observation lends support to the concept that they are all dependent on the same basic disturbance, namely, a circus contraction in the auricles.

10. Ectopic ventricular beats were seen after quinidin in all of the eight cases under discussion. These occurred at times in such fashion as to cause coupled rhythm.

11. After the continued administration of quinidin, paroxysms of ectopic ventricular tachycardia sometimes ensued. This arrhythmia is significant for the clinician; for in dogs injected with digitalis or strophanthin it is not infrequently the precursor of ventricular fibrillation and death. In the three cases in which this mechanism was seen, the paroxysms were fortunately of short duration.

12. In certain patients, quinidin is a toxic substance for heart muscle, even in doses ordinarily regarded as safe for clinical use. Carefully administered, the drug possesses great therapeutic virtue, whereas unintelligently given, it may be expected to cause undesirable effects.

APPENDIX.

REPORT OF CASES.

Cases in Which the Normal Mechanism Was Restored.

CASE 1.—Hosp. No. 3968. Male, aged 55. Diagnosis: Syphilis; chronic myocarditis. Known duration of fibrillation, one and a half years; duration of cardiac symptoms, two years.

This patient received quinidin sulphate on five different occasions, four times to restore the normal rhythm, the fifth time in an attempt to abolish auricular and ventricular premature beats at a time when the sinus rhythm prevailed.

First Course.—March 10 to 15: 4.6 gm. were given in six days. The patient had been taking 0.1 gm. digitalis (digitan) daily prior to quinidin administration. The original mechanism, as recorded electrocardiographically, was fine auricular fibrillation. There was left ventricular preponderance and an occasional left ventricular premature beat.

First Day: 0.2 gm. at 3 and 6 p. m. There was no evident effect.

Second Day: 0.2 gm. at 7, 9, 11 a. m. and 1 and 3 p. m. At 10:50 a. m., after 0.8 gm., the ventricular rate rose from 110 to 142 and right ventricular premature contractions appeared; these were transient and were not again observed during this course of treatment. By 4 p. m. the rate had again fallen to 95.

Third and Fourth Days: No quinidin given.

Fifth Day: 0.4 gm. at 12 noon, 4 and 8 p. m. Again there was acceleration of rate, which at 6 p. m. was 130.

Sixth Day: At 6 a. m., the ventricular rate was 140, with but 114 beats palpable at the wrist. 0.4 gm. at 8 and 10 a. m., 12 noon, 2:15 and 4 p. m. At 2:50 p. m., after 1.6 gm., there was impure flutter with ventricular rate of 115. This was followed, after another 0.4 gm., by restoration of the normal mechanism with rate of 80. The sinus rhythm was recorded one hour and fifty-five minutes after the onset of flutter. On the following day the T wave in Lead III, which had been upright, became inverted and auricular premature beats were present. The latter disappeared spontaneously after being present for four days. The P-R (conduction) time during the period of normal rhythm ranged from 0.13 to 0.16 second. The sinus rhythm prevailed for twenty-three days. On the twenty-fourth day fine auricular fibrillation with ventricular rate of from 120 to 135 suddenly reappeared. In the course of three days the T wave in all three leads assumed the opposite direction from that previously recorded. There were occasional left ventricular premature beats. On the twenty-fifth day the ventricular rate rose to 184 and remained at this level until quinidin was again administered five days later.

Second Course.—April 13: Three doses of 0.4 gm. each were given at two hour intervals. After 0.4 gm., right ventricular premature contractions appeared, but were transitory. After 0.8 gm. there was gradual fall in rate from 180 to

Case 1. Hosp No 3968

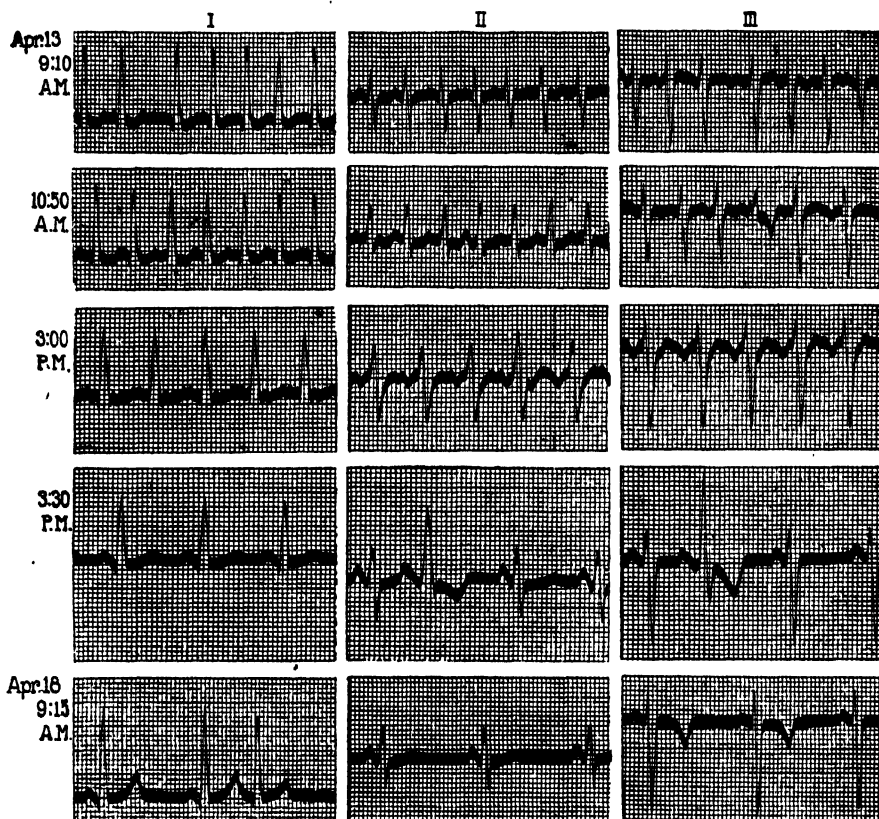


FIG. 1. Case 1. Hosp. No. 3968. April 13: Quinidin sulphate, 0.4 gm., at 10:30 a. m., 12 noon, and 2:30 p. m.

9:10 a. m., control. Auricular fibrillation; ventricular rate, 185; Q R S = 0.10 sec.

10:50 a. m., after 0.4 gm. Auricular fibrillation; ventricular rate, 185; right ventricular premature contraction (Lead III).

3:00 p. m., thirty minutes after 1.2 gm. Auricular flutter; auricular rate, 280; ventricular rate, 140. Q R S = 0.13 sec.

3:30 p. m., one hour after 1.2 gm. Normal rhythm; ventricular rate, 95; P-R = 0.13 sec. Q R S = 0.13 sec.

April 18, five days later. 9:15 a. m. Normal rhythm; auricular premature contractions; ventricular rate, 60; P-R = 0.13 sec.; Q R S = 0.10 sec.

140. The T wave in Lead I changed from inverted to upright. Three hours after beginning treatment and one hour after the second dose, there was impure flutter with ventricular rate of 145. The QRS interval was 0.10 second. Impure flutter persisted for forty minutes after which 2:1 flutter was recorded. The QRS interval was now from 0.12 to 0.13 second. After forty-five minutes of flutter, normal rhythm with rate of 95 was established. The T wave in Lead III changed at once from upright to inverted. Auricular and right ventricular

Case 1. Hosp. No. 3968

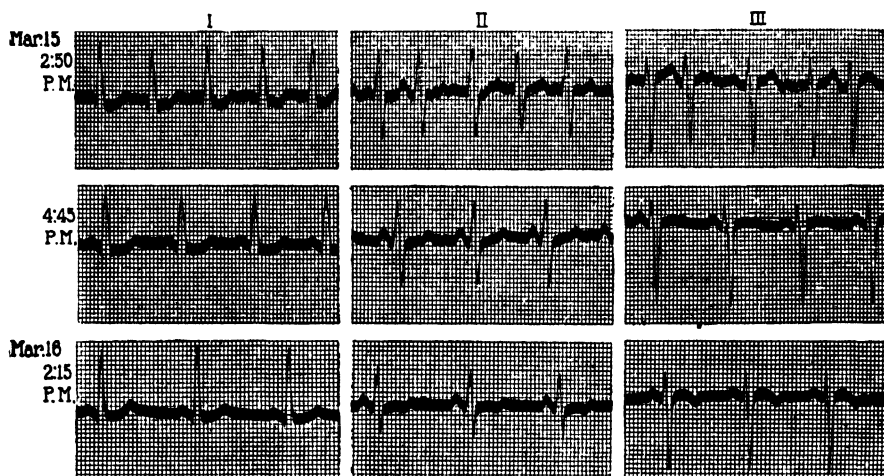


FIG. 2. Case 1. Hosp. No. 3968. March 15: Quinidin sulphate, 0.4 gm., at 8 and 10 a. m., 12 noon, 2:15 and 4 p. m.

2:50 p. m., after 1.6 gm. Auricular fibrillation; ventricular rate 115; T wave = $\approx$ + +.

4:45 p. m., forty-five minutes after 2 gm. Normal rhythm; ventricular rate 80; P-R = 0.15 sec.; T wave = + + +.

March 16, twenty-one hours later. 2:15 p. m. Normal rhythm; ventricular rate 69; P-R = 0.14 sec.; T wave = + $\approx$ -.

premature beats were present, the latter disappearing in the course of a few hours. The QRS interval still ranged from 0.12 to 0.13 second. Next day the rate was 69 and was maintained at about 70, until fibrillation reappeared twenty-one days after onset of normal rhythm. QRS group remained wider than normal, i.e., more than 0.10 second, for six days, then resumed its former value of 0.10 second. On the twenty-first day of normal rhythm fibrillation with ventricular rate of 120 set in. Next day there was further acceleration of rate, from 170 to 180, and the T wave in Lead III changed from inverted to upright.

Third Course.—May 6: 0.4 gm. sufficed to restore normal rhythm on this occasion, the ventricular rate falling rapidly from 185 to 94, and on the following day to 72. The T wave in Lead III again changed from upright to inverted and auricular premature beats appeared. Normal rhythm was present for only four days; then for a day fibrillation supervened, for a few hours there was spontaneous resumption of normal rhythm and then again reversion to fibrillation

Case 1. Hosp. No. 3968

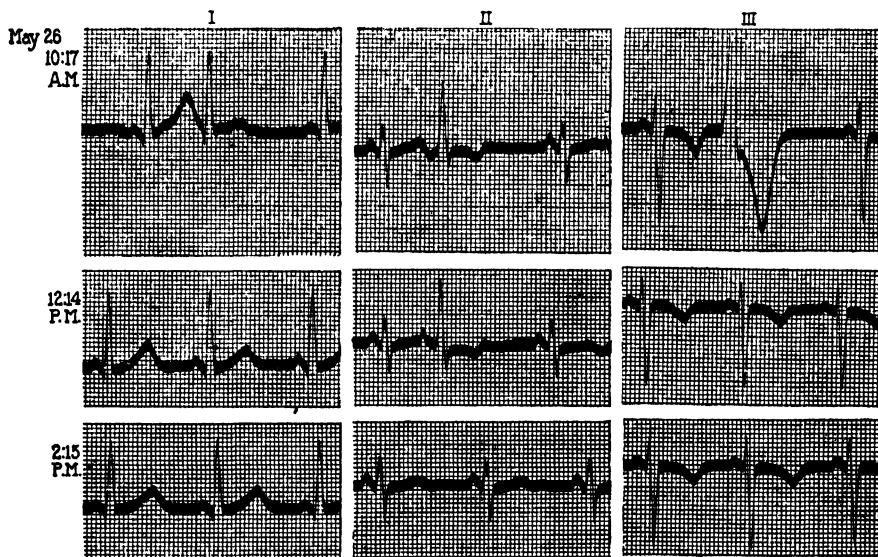


FIG. 3. Case 1. Hosp. No. 3968. May 26: Quinidin sulphate, 0.4 gm., at 8 and 11 a. m., and 1 p. m.

10:17 a. m., after 0.4 gm. Normal rhythm; auricular and right ventricular premature contractions; rate 75; P-R = 0.13 sec.

12:14 p. m., after 0.8 gm. Normal rhythm; auricular premature contractions; rate 66; P-R = 0.14 sec.

2:15 p. m., after 1.2 gm. Normal rhythm; rate 61; P-R = 0.12 sec.

with ventricular rate of 180. After the onset of fibrillation the T wave in Lead III again became upright. While the sinus rhythm prevailed, P-R (conduction) time was from 0.12 to 0.13 second.

Fourth Course.—May 18: After seven days of fibrillatory mechanism, 0.4 gm. was again given, and 2:1 flutter ensued forty minutes after quinidin was taken with no demonstrated intermediate mechanism. When flutter first appeared, the ventricular rate was 180 but fell in the course of thirty-five minutes to 145. One

hour and two minutes after the onset of flutter there was a normal rhythm at the rate of 108. There were occasional auricular premature beats. The T wave in Lead III changed from upright to inverted. At the end of four hours the ventricular rate had fallen to 75. P-R (conduction) time was from 0.12 to 0.14 second. Three days after the establishment of normal rhythm auricular flutter reappeared but the fibrillatory mechanism was not resumed. On the following day one dose of 0.4 gm. sufficed once more to restore the normal mechanism though occasional auricular premature beats were present.

Fifth Course.—May 26: In the course of four days many premature contractions, both of auricular and right ventricular origin, were present. These were sufficiently numerous to cause complaint; 1.2 gm., given in three doses of 0.4 gm. each, at three hour intervals sufficed entirely to abolish the ectopic contractions. The normal rhythm has now been maintained in this patient for more than eleven months. He has been taking 0.4 gm. quinidin every second day, and three doses of 0.4 gm. each on one day every two weeks. At times premature beats of auricular origin have been present, but the larger dosage has usually served to restore the undisturbed normal mechanism.

CASE 2.—Hosp. No. 4330. Male. aged 26. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency. Known duration of fibrillation, one month; duration of cardiac symptoms, eight years.

This patient received three courses of quinidin, each time with restoration of normal rhythm. He was kept in bed in the hospital for one month without medication before beginning quinidin administration. The original mechanism was coarse auricular fibrillation; there was right ventricular preponderance. Ventricular rate prior to treatment ranged from 100 to 110.

First Course.—March 17 and 18: 1.6 gm. given in two days.

First Day: 0.2 gm., at 11:30 a. m. and 1:30 p. m. The first effect noted was an increase in the rate of the ventricles to 125, even after this small preliminary dose. At 4:30 p. m., three hours after the second dose, there was auricular flutter with 3:1 block.

Second Day: 0.4 gm. at 7, 9 and 11 a. m. At 9:30 a. m., thirty minutes after the second dose, the rate was 140, and the mechanism auricular flutter with 3:1 block. There were a few left ventricular premature beats. One hour and five minutes after 1.2 gm. had been given, normal rhythm was established with a rate of 95. Premature beats were no longer present. The T wave in Lead III, which, prior to treatment, had been inverted, became upright in the course of two hours. There was a progressive fall in rate and at the end of five hours the ventricles were beating 73 times per minute. P-R (conduction) time was from 0.16 to 0.17 second. There were occasional auricular premature beats. The normal rhythm was maintained for thirteen days. It is of interest to note that on the last day of normal rhythm the T wave in Lead III again changed from upright to inverted, and the following morning auricular fibrillation was recorded once again, the T wave remaining as on the previous day. Ventricular rate was

Case 2. Hosp. No. 4330

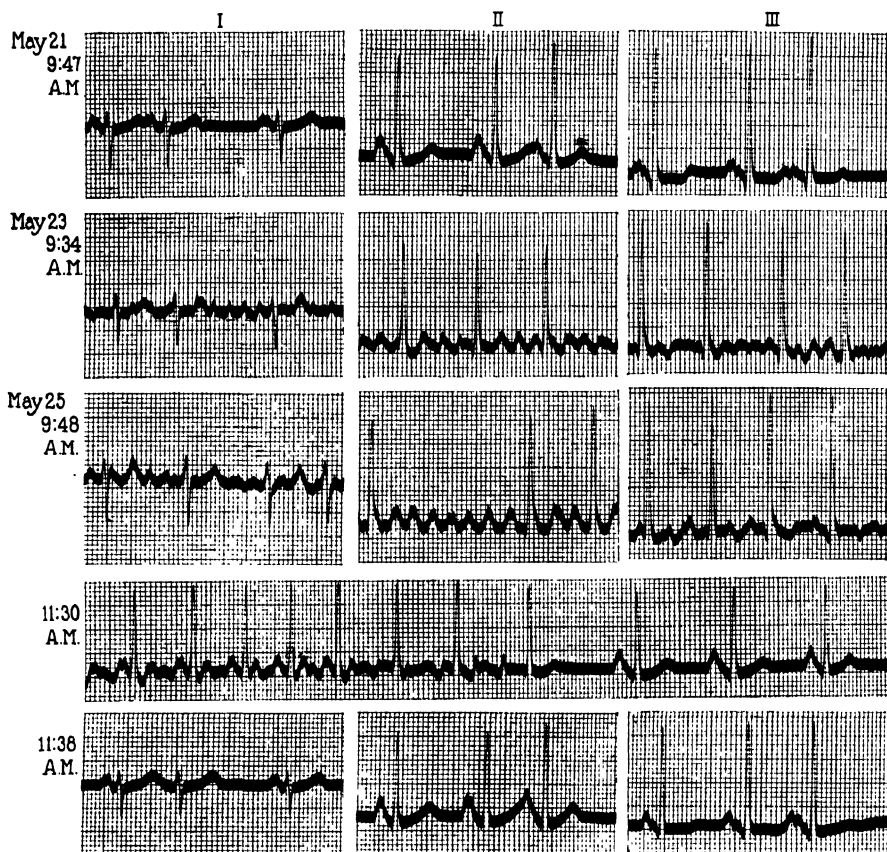


FIG. 4. Case 2. Hosp. No. 4330. Normal rhythm had prevailed for eighteen days after a previous course of quinidin. May 21: 9:47 a. m. Normal rhythm still present; rate 80; P-R = 0.15 sec.

May 23: 9:34 a. m. Auricular fibrillation and impure flutter; ventricular rate 92.

May 25: 9:48 a. m., control. Impure flutter and flutter; ventricular rate 90.
10:05 a. m., quinidin sulphate, 0.4 gm.

11:30 a. m., eighty-five minutes after 0.4 gm. Lead II. Transition from auricular flutter, with ventricular rate of 122 and auricular rate of 244, to normal rhythm, with ventricular rate of 72; P-R = 0.16 sec.

11:38 a. m. Normal rhythm; ventricular rate 86; P-R = 0.16 sec.

110. Two days later there was impure flutter with rate of 130, followed by coarse fibrillation with rate ranging from 110 to 115.

Second Course.—April 7: On the eighth day after reversion to fibrillation, 0.4 gm. was given at 7 and 9 a. m. The normal rhythm was again promptly reestablished at 9:30, the intermediate mechanisms, if such were present, not being recorded. The ventricular rate was 85. The T wave in Lead III again changed from inverted to upright, and auricular premature beats were present. These were observed to disappear and reappear at times. P-R (conduction) time was from 0.14 to 0.16 second. On this occasion the normal rhythm persisted for twenty-three days, the ventricular rate ranging from 66 to 80. On the twenty-third day there was again auricular fibrillation with a ventricular rate of 120 and the T wave in Lead III became inverted. Four days later the normal rhythm was resumed without medication, and persisted for twenty days, the P-R (conduction) time being from 0.15 to 0.16 second and the T wave in Lead III being upright. Again there were occasional auricular premature beats. On the twentieth day there was a mixture of auricular flutter and fibrillation, with ventricular rate of 92 and an inverted T wave in Lead III. On the following day there were seen in the same curve both impure flutter and auricular fibrillation, and on the day after this flutter, impure flutter and auricular fibrillation.

Third Course.—May 25: 0.4 gm. was given at 10:05 a. m. At 11 o'clock there was impure flutter, and at 11:30, one hour and twenty-five minutes after quinidin had been given, the transition from auricular flutter to the normal mechanism was recorded in Lead II. There were occasional auricular premature beats. T₃ was again upright. There was a fall in ventricular rate from 122 during the period of flutter, to 72 after the sinus rhythm was restored. The P-R (conduction) time ranged from 0.16 to 0.17 second.

The patient was discharged from the hospital three days later with a normal rhythm, but did not report for observation as directed. He returned for examination four months later showing the fibrillatory mechanism with a ventricular rate of 96. Three doses, of 0.4 gm. each, again restored the normal mechanism, and this was maintained for three months with the intermittent quinidin medication given on a plan similar in its essentials to that outlined for Case 1.

CASE 3.—Male, aged 55. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency. Known duration of fibrillation, fifteen months; duration of cardiac symptoms, six years.

The patient was partly digitalized prior to quinidin therapy, having taken some digitalis, the amount of which was not definitely known, before entering the hospital. He received four courses of quinidin. On two occasions only was the normal rhythm reestablished. The original mechanism was coarse auricular fibrillation, with a ventricular rate of 145.

First Course.—May 13: 1.2 gm. was given in one day. The patient received 0.4 gm. at 10:30 a. m., 12:30 and 2:30 p. m. There was no significant effect.

Case 2. Hosp. No. 4330

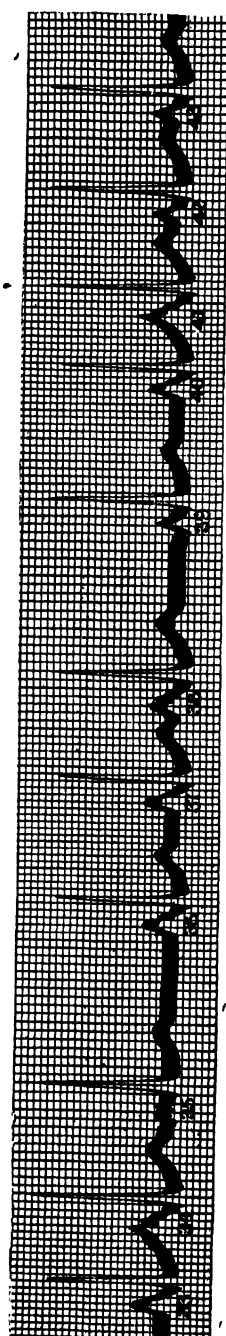
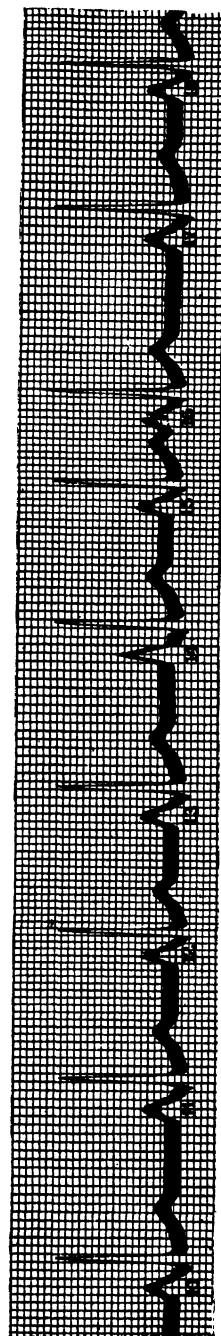
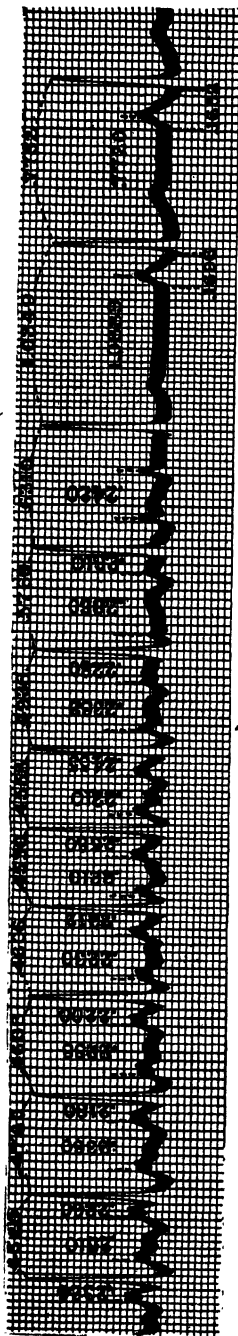


FIG. 5. Case 2. Hosp. No. 4330. Transition from auricular flutter to normal rhythm (Lead II) with tenth to eighteenth, and thirty-third to forty-third cycles after establishment of sinus rhythm. Occasional auricular premature contractions. (For tabulation of measurements, see Table II.)

Second Course.—May 19 to 25: 8.4 gm. were given in seven days. This course was given six days after the first attempt at therapy.

First Day: 0.4 gm. was administered at 10 a. m., 12 noon, 2 and 4 p. m. The ventricular rate before the first dose was 125, somewhat higher than when the first course was given. After 0.8 gm. this had risen to 150. After 1.2 gm. it

Case 3. Hosp. No. 4118

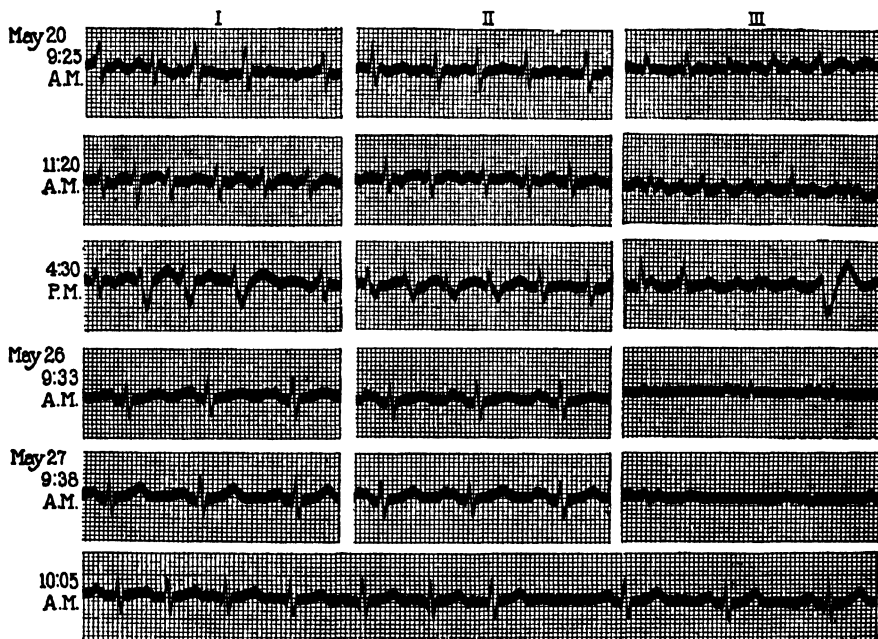


FIG. 6. Case 3. Hosp. No. 4118. May 20: Quinidin sulphate, 0.4 gm., at 10 a. m., 12 noon, 2 and 4 p. m.

9:25 a. m., control. Auricular fibrillation; ventricular rate 120.

11:20 a. m., after 0.4 gm. Coarser auricular fibrillation; ventricular rate 130.

4:30 p. m., after 1.6 gm. Auricular fibrillation; paroxysms of ventricular tachycardia; isolated left ventricular premature beats.

May 21 and 22: No quinidin given.

May 23 and 24: Quinidin sulphate, 1.6 gm., on each day.

May 25: Quinidin sulphate, 2 gm.

May 26: 9:33 a. m. Normal rhythm; ventricular rate 78; P-R = 0.24 sec.

May 27: 9:38 a. m. Normal rhythm; sino-auricular block; ventricular rate 80; P-R = 0.22 sec.

10:05 a. m. Paroxysm of auricular tachycardia with transition to normal rhythm.

was again 120 and there were premature beats of right ventricular origin. At 4:35 p. m., i. e., thirty-five minutes after a total of 1.6 gm. had been administered, there was ectopic right ventricular tachycardia with a rate of 120.

Second Day: The following morning ventricular tachycardia had disappeared and the ventricular rate was 120. Four-tenths gram was again given at 10 a. m., 12 noon, 2 and 4 p. m. At 11:20, one hour and twenty minutes after the first dose, there was impure flutter in addition to the coarse fibrillation. At 1:30, one hour and thirty minutes after the second dose, there was again auricular fibrillation without flutter, but there were right ventricular premature beats. The ventricular rate was 140. At 4:30, thirty minutes after the fourth dose, there were paroxysms of ventricular tachycardia originating first in the right ventricle, then in the left, together with isolated left ventricular premature beats; the rate was 130.

Third Day: There was again auricular fibrillation with a ventricular rate of 126. No drug was given on this day or on the fourth day of treatment.

Fifth Day: 0.4 gm. was given at 11 a. m., 1, 3 and 5 p. m. The ventricular rate at 9:54 a.m., before giving quinidin, was 145; at 4:15 p.m. it was 115. No change in mechanism was noted.

Sixth Day: At 9:27 a. m., there was impure flutter with rate of 130; 0.4 gm. was given at 10 a. m., 12 noon, 2 and 4 p. m. At 1:30 p. m., one hour and thirty minutes after a total of 0.8 gm., had been given, there were right ventricular premature beats. At 3:55 p. m., fifty-five minutes after the third dose, there were paroxysms of left ventricular tachycardia with a rate of 130. At 4:15 p. m. these had disappeared and there were isolated right ventricular premature beats.

Seventh Day: 0.4 gm. given at 9:30 and 11:30 a. m., 1:30, 3:30 and 6 p. m. Electrocardiograms taken on this day showed impure flutter with a ventricular rate ranging from 110 to 130. At about 7 p. m. the normal rhythm was established, the patient being distinctly conscious of the alteration in mechanism. The following morning at 9:33, this was recorded with a rate of 78, the P-R (conduction) time being 0.24 second. The normal rhythm persisted for fourteen days, during which the rate ranged from 75 to 90, and the P-R (conduction) time from 0.21 to 0.24 second. On the second day of normal rhythm an isolated instance of sino-auricular block was photographed, and later on this same day a short paroxysm of auricular tachycardia with the transition to the normal rhythm. On the twelfth and thirteenth days of normal rhythm there were auricular premature beats, and on the fourteenth day auricular fibrillation with rate of 102 was again present. The T wave during the time when normal rhythm was present showed no significant alteration. Fibrillation proceeded for three days, the ventricular rate during most of this period ranging from 150 to 160.

Third Course.—June 16 to 20: **First Day:** 0.4 gm. given at 10:30 a.m., 12:30 and 2:30 p. m. At 11:30 a. m., one hour after the first dose, there were right ventricular premature beats. At 1:40, one hour and ten minutes after the second dose, there were portions of the curves showing impure flutter in addition to fibrillation, with both right and left premature beats, the ventricular rate being 140. The ventricular premature beats disappeared before the third dose was given but were again present one hour after 1.2 gm. had been administered.

Second Day: Impure flutter with coarse fibrillation still prevailed; 0.4 gm. given at 10 a. m., 12 noon and 2 p. m. At 10:50, fifty minutes after the first dose, the curves showed flutter and impure flutter with a ventricular rate of 160. After the second dose there was again coarse fibrillation with right ventricular premature beats. One hour after the third dose there was again coarse fibrillation with impure flutter and right ventricular premature beats.

Third Day: No quinidin was given. The mechanism was impure flutter with rate of 145.

Fourth Day: No records were made.

Fifth Day: At 9:50 there was coarse fibrillation and impure flutter with a rate of 160; 0.4 gm. given at 11 a. m., 1, 3, 5 and 7 p. m. The rate remained at about this level. At 3:40, forty minutes after the third dose, there were occasional premature beats arising in both right and left ventricles. Fifty minutes later these were more numerous.

Sixth day: A record taken at 9:50 a. m. showed the normal mechanism with a rate of 88, the P-R (conduction) time 0.21 second and an upright T wave in Lead III, it having been previously inverted. At 4 p. m. on this day the rate was 85 and there were occasional auricular premature beats. The duration of normal rhythm was brief, and on the following morning there was again coarse auricular fibrillation with rate of 150 and flattening of the T wave in Lead III.

Fourth Course. June 22: 1.6 gm. were given in one day, 0.4 gm. at 10:30 a. m., 12:30, 2:30 and 4:30 p. m. At 11:30, one hour after administering 0.4 gm., there was, in addition to auricular fibrillation, impure flutter with rate of 180. One hour after the second dose, the rate had fallen to 142, and at 3:36, one hour and six minutes after the third dose, the rate was 125. There were at this time paroxysms of right and left ventricular tachycardia. At 4:25 these paroxysms were no longer noted, nor was impure flutter present. There was coarse auricular fibrillation with isolated right and left premature beats, the ventricular rate being 138.

On the following morning the ventricular rate was 134, and the patient complained of palpitation and dyspnea. He was accordingly given digitalis in the form of digitan, 1 gm. on this day and 0.5 gm. the following morning. No further attempt at quinidin therapy was undertaken.

Cases in Which the Restoration of the Normal Mechanism Was Not Accomplished.

CASE 4.—Female, aged 32. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency; aortic insufficiency. Known duration of fibrillation, five months; duration of cardiac symptoms, two years.

In five days 4.8 gm. was given. The original mechanism was auricular fibrillation, with right ventricular preponderance. The patient was well digitalized before beginning quinidin treatment, the ventricular rate ranging from 70 to 80.

First Day: Feb. 28: 0.2 gm. given at 11:50 a. m. and 5:30 p. m. There was no notable effect.

Second Day: 0.4 gm. given at 12:30 and 6 p. m. There was a sharp rise in heart rate to 142 at 8 p. m. with a gradual return to the former level of 64 the following morning.

Third Day: 0.4 gm. was given at 9:50 a. m., 2 and 6 p. m. Again there was a sharp rise in rate, reaching its maximum of 135 at 8 p. m., with return to 75 by the following morning. There were, in addition, occasional right ventricular premature beats which were first recorded three hours after the first dose on this day, and persisted throughout the night. The following morning the rate was 75.

Fourth Day: 0.4 gm. was given at 7:30 and 11:45 a. m., and 5 p. m. The rate again rose to 127 at 6 p. m. with gradual fall during the night. Right ventricular premature beats persisted.

Fifth Day: The rate in the morning was 82; 0.4 gm. was given at 8 and 11:30 a. m., and 5 p. m. Tachycardia again ensued, with maximum rate of 125 at 8 p.m. Premature beats became more numerous. No more quinidin was administered. The following morning the rate was again 69 to 74.

CASE 5.—Male, aged 19. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency; aortic insufficiency. Known duration of fibrillation, two months; duration of cardiac symptoms, eight years. The original mechanism was fine auricular fibrillation with right ventricular preponderance, the rate ranging from 63 to 80. During seven days 4.2 gm. was given.

First Day: Feb. 3: 0.1 gm. was given at 12 noon and 5 p. m. No effect was noted.

Second Day: 0.2 gm. given at 11:30 a. m. and 5 p. m. The rate remained 70. There was no significant change.

Third Day: 0.2 gm. at 10 a. m. and 4 p. m. There was a slight increase in heart rate to 104.

Fourth Day: The rate had returned to 60 but there were occasional right ventricular premature beats. No quinidin was given on this day.

Fifth Day: 0.4 gm. was given at 10:30 a. m. and 4 p. m. Three hours after the first dose, the rate was 110, the fibrillatory waves were slightly coarser and there were ectopic beats originating in both left and right ventricles. The T wave was unchanged. At 8 p. m. the ventricular rate was 126 but by the following morning it had fallen to 73.

Sixth Day: The fibrillatory waves were fairly coarse and the premature beats had disappeared; 0.4 gm. was given at 10:15 a. m., 1 and 4:55 p. m. At 11:35, one hour and twenty minutes after the first dose, the rate had risen to 91 and there were occasional right ventricular premature beats. The T wave in Lead III, which was upright, showed a higher voltage than before. At 12:20 the rate was 130, the curve showing auricular fibrillation and impure flutter, and more numerous right and left ventricular premature beats. At 4:25, three hours and twenty-five minutes after the second dose, the rate was 128, the rhythm flutter, and premature beats were present as before. At 4:45 there was again auricular fibrillation and impure flutter with a rate of 122, and at 5:55 reversion to coarse fibrillation with showers of right and left ventricular premature beats.

Seventh Day: The ventricular rate at 9:30 a. m. was 70. There was coarse fibrillation with occasional right and left ventricular premature beats; 0.4 gm. was given at 11:30 a. m., 1:30 and 3:30 p. m. There was a gradual increase in rate which at 6 p. m. was 111, but without alteration in mechanism. Coarse fibrillation and right and left ventricular premature beats persisted. On the following day, the tachycardia was still present, the ventricles beating at the rate of 131 at noon. There was also a sharp rise in temperature, which at 4 p. m. was 102 F. Whether or not this febrile reaction bore any relation to quinidin therapy it was not possible to say. At the time at which it occurred the patient

Case 6. Hosp. No. 4127

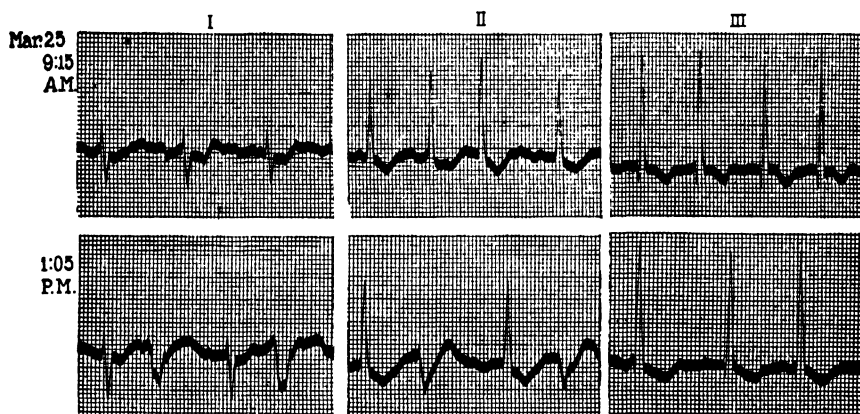


FIG. 7. Case 6. Hosp. No. 4127. March 25: Quinidin sulphate, 0.4 gm., at 7, 9, and 11 a. m.

9:15 a. m., after 0.8 gm. Auricular fibrillation; ventricular rate 78.

1:05 p. m., after 1.2 gm. Auricular fibrillation; ventricular premature contractions (Leads I and II), coupled with the usual ventricular complexes; ventricular rate 90.

complained of severe pain in the precordial region and in the left chest. The following morning the temperature was again normal, the ventricular rate 74. No more quinidin was administered.

CASE 6.—Female, aged 23. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency. Known duration of fibrillation, thirteen months; duration of cardiac symptoms, five years. The patient received two courses of quinidin.

First Course.—Feb. 24 to 28: 3.6 gm. was given in five days. The original mechanism was fine auricular fibrillation with right ventricular preponderance and occasional right ventricular premature beats. The rate ranged from 60

to 80. The patient had been receiving digitalis in the form of digitan for a number of days prior to quinidin therapy.

First Day: 0.2 gm. was given at 12 and 2 p. m. There was no notable effect.

Second Day: 0.4 gm. was given at 7, 9, and 11 a. m., and 1:10 p. m. Two hours and five minutes after the first dose there were numerous right ventricular premature beats which were rhythmically coupled with normal complexes. Fifty minutes after the fourth dose the extrasystoles disappeared and were not again recorded for two days. There was a slight increase in rate, from 78 to 90.

Third Day: The rate was again 74. There were no premature beats. No quinidin was given on this day.

Fourth Day: No quinidin was given.

Fifth Day: The rate at 9 o'clock was 62; 0.4 gm. was administered at 9:20 and 11 a. m., 1 and 3 p. m. At noon, one hour after the second dose, the ventricular rate was 94 and remained at a fairly constant level, between 80 and 90, thirty minutes after the fourth dose. At this time there were paroxysms of ventricular tachycardia with a rate of 115, and right ventricular premature beats of various forms. The paroxysms were still present at 5:45, and at this time left ventricular premature beats as well as others originating in the right ventricle were noted. By the following morning the ventricular rate was again 62 and the original mechanism, namely, fine auricular fibrillation, was present. No quinidin was given for eight days.

Second Course.—April 4 to 6: 3.2 gm. was given in three days.

First Day: 0.4 gm. was given at 11 a. m. and 4 p. m. At 3:15 the rate had risen to 145 and right ventricular premature beats were again present.

Second Day: 0.4 gm. was given at 7:15 and 11:15 a. m. and 4 p. m. The rate remained at a fairly constant level between 80 and 90, but at 4:15 right ventricular premature beats, which earlier in the day had not been present, reappeared.

Third Day: 0.4 gm. was given at 7:15 and 11:20 a. m. and 4 p. m. The rate at 8 a. m. rose to 117, fell gradually to 87 at 6 p. m., but on the following morning was again relatively high, namely, from 115 to 125. After a lapse of twenty-four hours, however, the ventricles were beating 73 times per minute. No more quinidin was given.

CASE 7.—Male, aged 58. Diagnosis: Chronic myocarditis. Known duration of fibrillation, one month; duration of cardiac symptoms, two years. This patient received 3.2 gm. quinidin in five days. The original mechanism was fine auricular fibrillation with left ventricular preponderance. He had been in the hospital prior to quinidin therapy for one month, during which time he received no medication. The ventricular rate during this time was quite labile, ranging from 90 to 120.

First Day: March 28: The rate at 9:45 a. m. was 120; 0.2 gm. was given at 10 a. m. and 12 noon. At 2:15 p. m. there were right and left ventricular premature beats, which by 4:20 had disappeared.

Second Day: The rate at 9:14 was 112; 0.4 gm. was given at 10 a. m., 12 and 2 p. m. At noon the rate had risen to 140 and there were paroxysms of left

ventricular tachycardia with isolated left ventricular premature beats. These paroxysms persisted throughout the day, becoming longer, so that at 5:30 they were almost continuous.

Third Day: No quinidin was given. The record at 9:25 a. m. showed 3:1 auricular flutter. At 10:40 the curve showed both auricular flutter and fibrillation. At 12:10 there was again pure flutter, with an occasional left ventricular premature beat, the ventricular rate ranging from 115 to 120.

Fourth Day: 3:1 flutter, with ventricular rate of 125, was still present at 9:07 a. m.; 0.4 gm. was given at 10:30 a. m. and 2 p. m. At 11:38 there was once again auricular fibrillation and flutter, and at 1:15, 2:1 flutter with ventricular rate of 145. This mechanism persisted throughout the day until 4 o'clock, at which time there was 2:1 flutter, with paroxysms of left ventricular tachycardia.

Fifth Day: At 9:05 a. m. the curve showed both impure flutter and fibrillation; 0.4 gm. was given at 10:10 a. m. and 2 p. m. At 11:30, one hour and twenty minutes after the first dose, there was 3:1 flutter with ventricular rate of 112. At 4:10, two hours and ten minutes after the second dose, there was 2:1 flutter with paroxysms of left ventricular tachycardia, ventricular rate being 132. A record made at 5:05 showed the persistence of this mechanism. On the following morning the flutter was still present, the ventricular rate being 120. Digitalis, in the form of digitan, 0.5 gm., was given at 10 a. m. and again at 11 a. m. At 11 o'clock there was auricular flutter and fibrillation, with a ventricular rate of 122. On the day following the administration of digitan, the ventricular rate was 80, and fine fibrillation, as it had appeared before beginning quinidin treatment, was again the prevailing mechanism.

CASE 8.—Male, aged 34. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency. Known duration of fibrillation, three weeks; duration of cardiac symptoms, one and one-half years. The original mechanism was fine auricular fibrillation. The patient had been in the hospital two weeks prior to quinidin therapy without medication, during which time the ventricular rate ranged from 90 to 110; 6.2 gm. quinidin was administered during an eight day period.

First Day: March 30: 0.2 gm. given at 2 and 4 p. m., with rise of ventricular rate from 94 to 105.

Second Day: At 9:26 a. m. the rate was 104 and there were occasional left ventricular premature beats; 0.4 gm. was given at 10:15 a. m., 12 noon and 2 p. m. At 11:30 the fibrillatory waves had become somewhat coarser and the rate was 110; at 2:05 the rate was 135. At 4:15 p. m. there was impure flutter with a ventricular rate of 120.

Third Day: At 9:16 a. m. the record showed coarse fibrillation and impure flutter with a ventricular rate of 112. The T wave in Lead II, which had previously been upright or diphasic, was now iso-electric; 0.4 gm. was given at 10:25 a. m., 12 noon and 2 p. m. At 11:52 there was impure flutter with right ventricular premature beats, and this persisted throughout the day, the ventricular rate ranging from 90 to 120. The T waves in Leads II and III were variable.

Fourth Day: At 9:42 the ventricular rate was 90 and there was coarse auricular fibrillation. The T wave in Lead III, which had before been inverted, was now upright. Only one dose of quinidin, 0.4 gm., was given at 10 a. m. because of complaint by the patient of epigastric discomfort.

Fifth Day: No quinidin was given.

Sixth Day: The ventricular rate was 120; 0.4 gm. was given at 7:30 and 11:45 a. m., and 5 p. m. There was no significant change in mechanism but

Case 7. Hosp. No. 4327

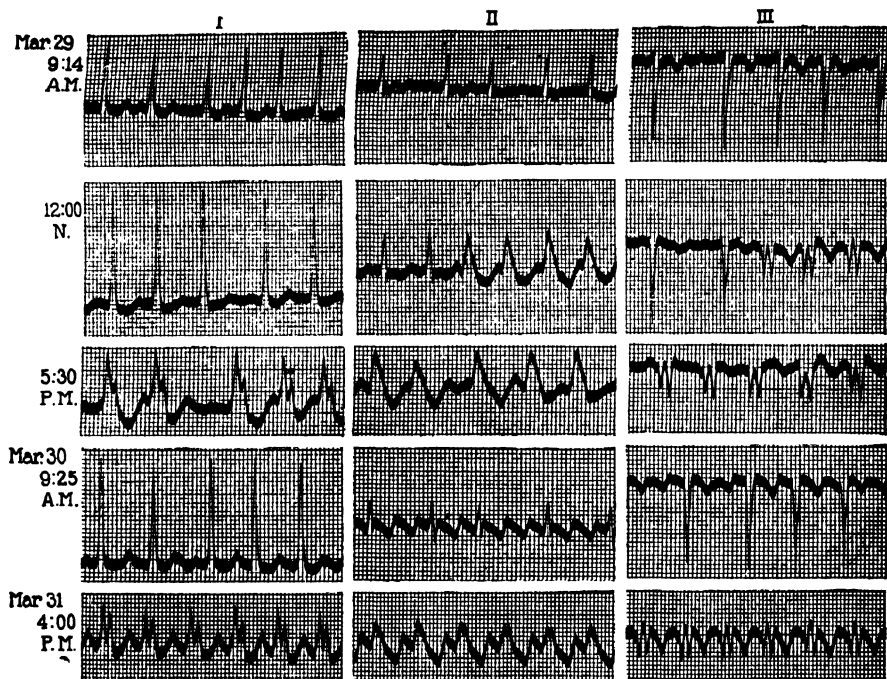


FIG. 8. Case 7. Hosp. No. 4327. March 29: Quinidin sulphate, 0.4 gm., at 10 a. m., 12 noon and 2 p. m.

9:14 a. m., control. Auricular fibrillation; ventricular rate 112.

12:15 p. m., after 0.8 gm. Auricular fibrillation; paroxysms of left ventricular tachycardia.

5:30 p. m., after 1.2 gm. Same as 12 noon. Longer paroxysms of tachycardia.

March 30: No quinidin given.

9:25 a. m. Auricular flutter; ventricular rate 115; auricular rate 345.

March 31: Quinidin sulphate, 0.4 gm., at 10:30 a. m. and 2 p. m.

4:00 p. m., after 1.2 gm. Auricular flutter; paroxysms of left ventricular tachycardia; ventricular rate 135; auricular rate 270.

rate fell gradually to 90 at 3:35 p.m. The T wave in Lead III again was variable in its direction.

Seventh Day: The ventricular rate was 115, the records showing coarse fibrillation; 0.2 gm. was given at 10:10 a. m., 2, 4, 6 and 8 p.m. This smaller and more fractionated dosage produced no notable effects.

Eighth Day: The ventricular rate was 120. There was coarse fibrillation; 0.4 gm. was given at 10:15 a. m. and 12 noon. There was no alteration in mechanism. Headache, slight nausea, sweating and palpitation induced discontinuation of therapy.

CASE 9.—Female, aged 33. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency. Known duration of fibrillation, two months; duration of cardiac symptoms, seven years.

This patient received 2.4 gm. quinidin in three days. The original mechanism was moderately coarse auricular fibrillation with occasional left ventricular premature beats. The ventricular rate prior to therapy ranged from 95 to 140.

First Day: March 31: 0.2 gm. was given at 11:30 a. m. and 12:30 p.m. There was no noteworthy effect.

Second Day: The rate at 9 a.m. was 112. No ventricular premature beats were recorded; 0.4 gm. was given at 10:20 a.m., 12 noon and 2 p.m. At 12 o'clock the rate had risen to 130 and there were numerous left ventricular premature beats. At 2:45 impure flutter was present, with a ventricular rate of 170. At 4:45 there was impure flutter and coarse fibrillation, with a ventricular rate of 110.

Third Day: The ventricular rate at 9:12 a.m. was 155, with occasional left ventricular premature beats; 0.4 gm. was given at 10 a.m. and 12 noon. There was no alteration in mechanism. The patient complained of severe headache and of precordial pain. On the following day digitan, 0.5 gm., was given at 11:30 a. m. and again at 12:30 p. m. Twenty-four hours later the ventricular rate was 62. Electrocardiograms showed a finer fibrillation than had previously been noted at any time.

CASE 10.—Female, aged 46. Diagnosis: Chronic endocarditis (rheumatic); mitral stenosis and insufficiency; aortic insufficiency. Known duration of fibrillation, three weeks; duration of cardiac symptoms, four months. The original mechanism was fine auricular fibrillation. This patient received three courses of quinidin. Prior to the first course her heart had been brought fairly well under the influence of digitalis, the ventricular rate ranging from 78 to 100.

First Course.—April 5 to 8: Three gm. was given in four days.

First Day: The ventricular rate at 9:45 was 90; 0.2 gm. was given at 12 and 3 p.m. At 4:55 the ventricular rate was 110 and there were right ventricular premature beats.

Second Day: 0.2 gm. was given at 7:30 a. m., 12 noon and 4:30 p. m. There was no noteworthy change in rate or mechanism.

Third Day: 0.4 gm. was given at 7:30 and 11:30 a. m. At 9:19 ventricular rate was 110 and the T wave in Lead I, which had previously been upright, was inverted; at 4:25, the rate was 120.

Fourth Day: The ventricular rate at 9:08 a. m. was 100; 0.4 gm. was given at 10:35 a.m., 12:30 and 2:45 p. m. There was a sharp rise in heart rate to 140 at 2:10 p.m., with gradual fall after 4 p.m. to 84 the following morning. The patient at this time was distinctly uncomfortable. She was dyspneic, the liver was large and tender, there was epigastric discomfort, and there had been, during the ten days preceding, a gradual gain in weight. Therefore during the next two days she was given a course of digitalis therapy with prompt disappearance of symptoms and improvement in signs. The heart rate fell to about 70 and there was no pulse deficit. Small doses of digitalis were continued thereafter for sixteen days.

Case 10. Hosp. No. 4349

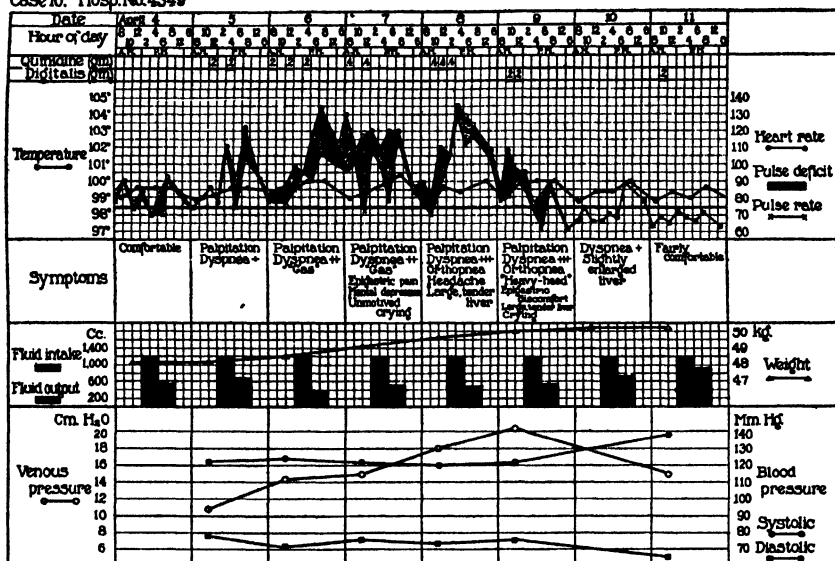


FIG. 9. Case 10. Simple tachycardia induced by quinidin was followed by signs and symptoms of heart failure. Digitalis afforded prompt relief.

Second Course.—April 27 to 29: On the seventeenth day after the first course was completed, the second course began; 1.6 gm. was given during a three day period.

First Day: The ventricular rate ranged from 60 to 70; 0.2 gm. was given at 12:25 and 2 p. m. There was no noteworthy effect.

Second Day: No quinidin was given.

Third Day: The ventricular rate was 55; 0.4 gm. was given at 10:30 a. m., 2:30 and 4:30 p.m. At 4 p.m. there were occasional right ventricular pre-

mature beats. At 6 p. m. the rate had risen to 132, but fell gradually during the night to its former level of 60 to 70. For three days no more quinidin was administered.

Third Course.—May 2 to 6: Four grams was given during a five day period, and at the same time the patient received 0.1 gm. digitan each day.

First Day: The ventricular rate was 54. The mechanism was fine fibrillation as before; 0.4 gm. was given at 10 a. m., 12 noon and 2 p. m. Once again there was a sharp rise in the ventricular rate to 132 at 4 p. m., with gradual subsidence of the tachycardia and resumption of a rate of 50 the following morning.

Second Day: No quinidin was given.

Third Day: 0.2 gm. was given at 10 a. m., 12 noon and 2 and 4 p. m. No significant effect in mechanism or rate ensued.

Fourth Day: The ventricular rate was 62; 0.2 gm. was given at 10:30 a. m., 12:30, 2:30, 4:30 and 6:30 p. m. Again, there was no change in rate or mechanism. The T wave in Lead I, which previously had been upright, was now iso-electric.

Fifth Day: The ventricular rate was 62; 0.2 gm. was given at 10:30 a. m., 12:30, 2:30, 4:30 and 6:30 p. m. As before this greater fractionation of dosage caused no effect on rate or mechanism.

CASE 11.—Male, aged 55. Diagnosis: Chronic myocarditis. Known duration of fibrillation, seven months; duration of cardiac symptoms, one and one-half years. The patient was well digitalized before beginning therapy. He received two courses of quinidin. His ventricular rate prior to quinidin administration ranged from 66 to 75. The original mechanism was fine auricular fibrillation with left ventricular preponderance.

First Course.—June 1 to 3: 3.2 gm. was given in three days.

First Day: 0.2 gm. was given at 1:40 and 3:40 p. m. There was no demonstrable effect.

Second Day: 0.4 gm. was given at 11 a. m., 1 and 3 p. m. There was no significant effect.

Third Day: 0.4 gm. was given at 10 a. m., 12 noon, 2 and 4 p. m. There was a slight rise in the ventricular rate, from 80 to 108 at 6 p. m. No drug was given during the next two days.

Second Course.—June 6 to 10: 6.8 gm. was given in five days.

First Day: 0.4 gm. was given at 10 a. m., 12 noon, 2 and 4 p. m. There was a slight rise in ventricular rate, from 74 to 94.

Second Day: 0.4 gm. was given at 10 a. m., 12 noon, 2 and 4 p. m. The ventricular rate rose from 82 at 9:50 a. m. to 105 at 3 p. m., and there were a number of left ventricular premature beats. At 4:30 p. m., thirty minutes after the fourth dose, there were paroxysms of left ventricular tachycardia in addition to coarser fibrillatory waves in the remainder of the electrocardiograms and a number of isolated left ventricular premature beats.

Third Day: Paroxysms of ventricular tachycardia were not present in the morning. The ventricular rate was 88; 0.4 gm. was given at 9:30 and 11:30

a. m., 1:30 and 3:30 p. m. At 12:33, one hour and three minutes after the second dose, there were again paroxysms of left ventricular tachycardia with a rate of 100, and isolated left ventricular premature beats. The same mechanism was again present at 4:30 p. m. with a rate of 92.

Fourth Day: Paroxysms of tachycardia no longer were present. There were occasional left ventricular premature beats. The ventricular rate was 82; 0.4 gm. was given at 11 a. m., 1, 3 and 5 p. m. There was a distinct rise in the ventricular rate, which at 8 p. m. was 128. Left ventricular premature beats persisted.

Fifth Day: The ventricular rate was 104; 0.4 gm. was given at 11:20 a. m. At 2 p. m. the ventricular rate was 118 and the patient was decidedly uncomfortable. He complained of palpitation, some precordial pain, heaviness of his head and of vague epigastric discomfort. His temperature at this time rose to 101 F. At 2:15, therefore, digitalis therapy was begun, with prompt reduction in rate to 69 the following day, with subsidence of fever and relief of symptoms.

CLINICAL STUDIES OF QUINIDINE.*

III. SOME RESULTS OF THE QUINIDINE TREATMENT OF AURICULAR FIBRILLATION.**

By ROBERT L. LEVY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

About 40 per cent of the cardiac irregularities are due to fibrillation of the auricles. In about half of these cases, the arrhythmia is associated with chronic valvular heart disease affecting the mitral orifice, particularly mitral stenosis. In approximately one quarter of the cases there is found evidence of myocardial disease without valve involvement. The remainder comprise a group in which are seen a relatively small number of individuals with aortic disease and a somewhat larger number in which the nature of the heart affection is undetermined.

A majority of patients with auricular fibrillation complain of unpleasant symptoms which are directly related to the presence of the irregularity. The more common abnormal sensations experienced are palpitation, precordial oppression and epigastric discomfort. Other symptoms of heart failure may be added, of which the number and severity are, in general, proportional to the ventricular rate. The number of ventricular contractions may range from 60 to 180 per minute, and is commonly above 90. The rhythm is perpetually irregular and many ventricular contractions are ineffectual in propagating a pulse wave to the wrist. This gives rise to the *pulse deficit*. In order to maintain such individuals in

* Read at the Annual Meeting of the Medical Society of the State of New York, at Albany, April 20, 1922.

** The first two papers in this series were:

I. Restoration of the Normal Cardiac Mechanism in Auricular Fibrillation by Quinidine, *Jour. Amer. Med. Assn.*, 1921, lxxvi, 1289.

II. Alterations in the cardiac mechanism after administration of quinidine to patients with auricular fibrillation, with consideration of certain toxic effects of the drug. *Arch. Int. Med.*, 1922, xxx, 451.

relative comfort, it is necessary, by continuous digitalis medication, to keep the heart rate at or about 70. Their response to effort is limited because tachycardia is readily induced and is accompanied by an increase in the number of beats which fail to reach the peripheral circulation. On the other hand, many individuals with auricular fibrillation, properly cared for, lead fairly comfortable and useful lives for a number of years.

It is not an overstatement to assert that the restoration of the normal cardiac rhythm by quinidine sulphate in patients with auricular fibrillation is one of the most dramatic pharmacotherapeutic accomplishments in clinical medicine. Furthermore, this drug is unique in its action in that it serves to restore to normal a disturbed physiological mechanism.

The Drug.

Quinidine is obtained from cinchona bark as a by-product in the manufacture of quinine, of which it is the dextrorotatory stereoisomer. It was at one time regarded as a cheap substitute for quinine, but was discarded as inferior in the treatment of malaria. Quinidine sulphate, $(C_{20}H_{24}O_2N_2) \cdot H_2SO_4 + 2H_2O$, which is employed in the clinic because of its greater solubility, occurs as minute, silky, white crystals. It is odorless, bitter, soluble in water, and is best administered in gelatin capsules.

The Mechanism of Auricular Fibrillation and the Action upon It of Quinidine.

The older theory of fibrillation regarded this disturbance in rhythm as due to the presence of multiple irritable foci in the auricle, which gave rise to a battery of impulses occurring in rapid succession, of which a variable number were transmitted through the auriculo-ventricular bundle to the ventricle. The latter responded with contractions of inconstant force and irregular rhythm. More recently there has arisen a newer concept, developed from the work of Garrey and of Mines, and amplified by Lewis. According to these investigators, a *circus contraction*, having its path about the mouths of the great veins, is the mechanism which is at the basis of both fibrillation and flutter of the auricles. A circus contraction has actually been

demonstrated in the fibrillating auricle of the dog and has been shown to exist in man by the application of trigonometric formulæ to the human electrocardiogram.

Quinidine prolongs significantly the refractory period of the auricular muscle and so tends to terminate the circus contraction. On the other hand, the drug also retards fiber conduction in the auricle, an effect favoring continuation of the circus. Normal rhythm probably follows its administration when the first effect predominates over the second.

Clinical Experience with Quinidine.

Since the introduction of quinidine into clinical therapeutics by Frey in 1918, reports of more than 300 cases of auricular fibrillation treated with this drug have appeared in the literature. In from 40 to 50 per cent of patients treated, it has been possible to restore the normal rhythm for varying periods of time. Since the more important points relating to the results of therapy may be illustrated from personal experience, the following discussion will be based largely upon a consideration of the first 25 patients with auricular fibrillation treated with quinidine in the Hospital of the Rockefeller Institute.

These 25 cases represent an unselected material. The group comprises 11 cases of mitral stenosis, 7 cases of combined mitral and aortic valvular disease and 7 cases without demonstrable valve lesion. In 11 cases (44 per cent) the normal rhythm was restored.

Before attempting to alter the cardiac rhythm, it is well to combat heart failure, if this be present. Rest in bed, restricted fluid intake, a properly regulated diet, and digitalis, if necessary, will usually serve to build up a cardiac reserve power.

In administering quinidine, it has been found advisable to give the individual doses at intervals of 2 hours. For the drug is rapidly excreted and the greater the fractionation of dosage, the more rapidly is it eliminated from the body. The patient should remain in bed, under careful observation by nurse and physician and preferably in a hospital where graphic records can be made at frequent intervals. The reason for urging these precautions will appear from the subsequent discussion.

A satisfactory plan of treatment is as follows: *1st day*: 2 doses of 0.2 gm. (3 grains). These suffice to test for an idiosyncrasy to members of the cinchona group; *2nd day*: 3 doses of 0.4 gm. (6 grains); *3rd day*: 4 doses of 0.4 gm. (6 grains); *4th day*: 5 doses of 0.4 gm. (6 grains); *5th day*: 5 doses of 0.4 gm. (6 grains). Not more than 2.0 gms. (30 grains) are given in a 24 hour period. It is rarely necessary to continue therapy after the fifth day, for if normal rhythm has not ensued after 5 days of treatment, it will probably not result from further dosage; or, if it should appear, will persist for but a short period of time.

Cases in Which the Normal Rhythm Was Restored.—The first effect of quinidine is usually acceleration of ventricular rate. The action of any single dose is transitory, usually passing off in the course of 2 to 4 hours. Occasional premature beats are also not uncommonly observed. The auricular rate is slowed and auricular flutter is often the transitional mechanism between fibrillation and the normal rhythm. Regularization occurs for the most part on the second to the fourth day of therapy, and patients are commonly aware of the change when the rhythm alters. The total amount necessary has varied from 0.4 gm. to 8.4 gms. given over a period ranging from 1 to 7 days. The average dose has been 2.6 gms., given in a 3 day period.

Four patients who have fared extremely well have been instances of myocardial disease, without valve lesion and with but slight cardiac enlargement. It has been maintained by most observers that short duration of fibrillation predisposes to a favorable outcome. Yet the following instance indicates that even when the arrhythmia has been known to exist for a period of years, the sinus may resume activity and a normal rhythm ensue.

J. W. H., Hosp. No. 4429, male, age 43. He had several attacks of malaria, and one attack of dysentery. He had syphilis 21 years ago and received thorough treatment both with mercury and iodide, and salvarsan. The Wassermann reaction on the blood serum was negative on admission to the hospital. His cardiac symptoms first appeared 10 years ago, fibrillation 7 years ago. He had taken no digitalis. His chief complaint was "palpitation."

Examination revealed a large man. The heart shadow in the teleroentgenogram extended 11.5 cm. to the left, 5 cm. to the right.

There were no cardiac murmurs. The average ventricular rate was 80. The blood pressure was 140 mm. Hg. systolic; 96 diastolic.

He was given 3.2 gm. of quinidine in 3 days, according to the plan previously outlined, and on the evening of the 3rd day, after slight tachycardia, with maximum ventricular rate of 104, normal rhythm with rate of 83, was established. The patient returned to his home in Kansas City and was actively at work in the real estate business for 3½ months, during which time the normal mechanism prevailed without further medication. At the end of this period, there was sudden reversion to fibrillation. He returned to the hospital for further treatment. The ventricular rate was 80 to 90. After 3.2 gm. of quinidine, normal rhythm was again restored, the ventricular rate averaging 70 per minute. He is again at work.

In women with heart disease, especially when mitral stenosis is present, the strain of labor is often poorly borne. Fibrillation of the auricles may be induced, with accompanying symptoms of heart failure. In such a patient an astonishingly small amount of quinidine served to bring fibrillation to an end.

A. J. N., Hosp. No. 4393, female, age 32. The patient was admitted to the hospital complaining of shortness of breath and swelling of the legs and abdomen. She had growing pains during her 13th and 14th years. There was no history of sore throats or definite rheumatic infection. She had known for 5 years that she had heart trouble, but the rhythm was regular.

The present illness began with the birth of her child, 6 weeks before admission to the hospital. She had felt fairly well during pregnancy. Labor began at the end of 7½ months and after it had lasted 24 hours, the baby was taken from her with low forceps under ether. She noticed immediately after the birth of the child that she was somewhat short of breath. She was kept in bed for 3 weeks and given intermittent courses of digitalis. She then remained at home for 3 weeks, doing her housework up to the time of admission to the hospital.

On examination there was dyspnea, orthopnea and cyanosis. There were signs of a large right hydrothorax and moderate ascites. There was marked edema of the feet and lower legs. The heart was displaced to the left, but not markedly enlarged. The auscultatory findings were characteristic of mitral stenosis and auricular fibrillation.

After 2 weeks rest in bed, restricted fluid intake and removal, in three aspirations, of 3,300 cc. from the right pleural sac, the edema was gone and the symptoms relieved. The heart rate at this time averaged 95, but the rhythm was still irregular. She was given 0.2 gm. of quinidine at 11 A. M. and again at 1 P. M. At 4 P. M. an electrocardiogram still showed fibrillation. The following morning, the normal mechanism, with rate of 88, was recorded.

This patient has now been under observation for 6 months, during which the sinus rhythm has persisted without further medication. She is able to perform her household duties without effort.

The duration of normal rhythm after a single course of treatment has been variable, ranging from a few hours to more than 6 months. In one man with syphilitic myocarditis, in whom the duration of normal rhythm after one course of therapy was from 20 to 23 days, the sinus mechanism has been maintained, with one brief interruption, for over 10 months, by small daily doses. Having once learned a patient's tolerance for the drug, cumulative effects need not be feared; for quinidine is rapidly excreted.

Unpleasant symptoms due to quinidine have been few in patients in whom the normal mechanism has been restored. In the literature are contained reports of several instances of embolism following establishment of regular rhythm, and the suggestion has been made that they were due to the expulsion of small bits of clot from one of the auricles. It must be remembered that embolism not infrequently occurs in heart disease with fibrillation when not treated with quinidine. The accident is always unfortunate, sometimes fatal, but is unavoidable. The following is such a case:

K. C., Hosp. No. 4504, female, age 44, was admitted to the hospital complaining of shortness of breath and weakness. She had frequent attacks of tonsillitis and 3 attacks of chorea between the ages of 6 and 14. Eight years ago the tonsils were removed. She had symptoms referable to the heart for two years and it is probable that she was suffering from fibrillation of the auricles during this period.

Examination showed no dyspnea at rest, and no edema or cyanosis. The cardiac dulness extended 12.5 cm. to the left in the 5th space, 4.5 cm. to the right in the 4th space. The auscultatory findings were those of mitral stenosis. She had been taking digitalis before coming

to the hospital; the ventricular rate was 80. The blood pressure was 112 mm. Hg. systolic; 82 diastolic.

She was kept in bed and given in all, 0.5 gm. digitalis in the form of digitan. On the 10th day of hospitalization, quinidine treatment was begun. During a 6 day period, 6.8 gm. were administered. The patient complained at various times of headache, tinnitus aurium and slight epigastric discomfort. There was slight acceleration of ventricular rate on each day. At 8 P. M. on the 6th day, normal rhythm was observed by the nurse to be present. At 8.40, quite suddenly the patient fell unconscious to the floor. After being put back to bed, she had a generalized convulsion, involving both arms and legs. She became very noisy, restless and difficult to manage. When examined at 9.30 she was still unconscious and quiet. There were no obvious signs of a local cerebral lesion. At 10.15 there was another convulsive seizure. By 1.30 the following morning, consciousness had returned. She vomited twice, and then slept during most of the remainder of the day.

Normal rhythm, with rate of about 90, persisted for 36 hours. Then fibrillation recurred, with ventricular rate of 126, 117 beats coming through to the wrist. Digitalis served promptly to control the heart rate.

It is probable that the attack of unconsciousness with convulsions was due to a small cerebral embolus, which was whipped off from a thrombus in the left auricle. Fortunately it lodged in a silent area in the brain and left no residual effects.

Several sudden and unexplained deaths have been reported by competent observers following the administration of quinidine. It is possible that some of these fatalities may have been due to the occurrence of ventricular tachycardia, and ventricular fibrillation, to which reference will be made later. On the other hand, as in the one instance which we have observed, clinical observation and necropsy may fail to reveal the reason for exitus.

J. P., Hosp. No. 4511, female, age 49, was first seen in October, 1919, complaining of shortness of breath and swelling of the legs and abdomen. She had frequent sore throats and had influenza in the 1918 epidemic. Symptoms referable to the heart had been present for 3 years. Edema was first noticed 4 days before admission to the hospital.

Examination revealed general anasarca, an enlarged heart, auricular fibrillation, but no evidence of valvular disease. The blood pressure was 144 mm. Hg. systolic; 84 diastolic. She responded well to the usual forms of therapy, including digitalis. The tonsils were large and badly infected, and were removed under local anesthesia. She left the hospital free from symptoms, still taking digitalis in order to maintain a slow ventricular rate.

The patient was not seen for $2\frac{1}{2}$ years. During this time she did light housework and took occasional doses of digitalis at irregular intervals. Slight exertion caused dyspnea and edema of the legs was present almost constantly.

She was readmitted to the hospital on April 3rd, 1922. At rest she was fairly comfortable. The lungs were clear. The heart was enlarged, dulness extending 15.5 cm. to the left in the 5th space, 5.5 cm. to the right in the 4th space. The rhythm was totally irregular. There was a systolic murmur at the apex, transmitted to the axilla. The pulmonic second sound was accentuated. The liver edge was felt just below the costal margin. The blood pressure was 156 mm. Hg. systolic; 82 diastolic. There was brawny edema of the ankles, legs and buttocks.

With rest, limited fluid intake and digitalis, there was marked loss of weight from 70.6 to 65.8 Kg. in 3 days. The edema disappeared. The heart rate averaged 71, with 67 beats felt at the wrist.

On April 11th, she received quinidine, 0.2 gm. at 11 A. M. and 1 P. M. There was slight acceleration of ventricular rate to 98 at noon. The patient complained of flushing, palpitation and dizziness. On April 12th, the heart rate at 8 A. M. was 68; these symptoms had disappeared. She was given quinidine, 0.4 gm. at 12 noon, 2 and 4 P. M. Again there was tachycardia, the heart rate at 6 P. M. being 100, with 74 beats at the wrist. There was no alteration in rhythm. She complained of flushing, headache, palpitation and nausea. She passed a comfortable night. On April 13th, all symptoms had disappeared; the heart rate was 77. Quinidine 0.4 gm. was given at 11.30 A. M., 1.30, 3.30 and 5.30 P. M. There was slight increase in heart rate, with maximum of 114 per minute at 2 P. M. There was some flushing after the second dose. At 6 P. M. there was headache. The patient passed a fairly comfortable night. She slept, however, only three or four hours, according to the nurse's chart.

On April 14th, at 6 A. M., the nurse noted that the heart rhythm was regular, 69 to the minute, with no pulse deficit. The patient was seen at 7.35, when she said she felt very well but noted that her heart gave an occasional jump. At 7.38, the nurse re-entered the room, found her moaning, with face markedly cyanotic, hands clenched, pulse not perceptible and respirations deep and sighing. At 7.40 A. M. respirations ceased.

Necropsy was performed four hours after death. The heart was markedly dilated, especially the right auricle. No ante-mortem clots were found in any of the chambers, the walls of which were quite smooth. The mitral valve was a little puckered in places, but there were no organized or fresh vegetations on any of the valve leaflets. There was definite hypertrophy, especially of the left ventricle. The heart weighed 400 gms. The coronary arteries were patent and contained no thrombi or emboli.

The lungs were emphysematous. The pulmonary arteries were carefully dissected out to their small ramifications, but no pulmonary embolus was discovered.

The other necropsy findings were irrelevant to the present discussion. Permission to examine the brain was not obtained.

The question is often asked, "Are patients in whom regular rhythm has been restored better off than they were as fibrillators?" When the normal mechanism can be maintained, the answer is an unqualified "Yes." They are better because consciousness of the heart's action and the sense of substernal oppression have disappeared; they are better because it is no longer necessary continually to take digitalis, as patients with fibrillation usually must in order to maintain a slow ventricular rate; they are better because the response of the heart to effort is better regulated; and they are better because they know that the heart rhythm is regular, like that of a normal individual.

Cases in Which Restoration of the Normal Rhythm Was Not Accomplished.

This group of 14 cases was distinguished clinically by the fact that in all there was conspicuous cardiac enlargement. This may be taken as evidence either of serious derangement of cardiac mechanics by valvular disease or of extensive myocardial involvement, or per-

haps of both. The average duration of symptoms and the known duration of fibrillation in these patients did not differ materially from the figures for the first group. The amount of quinidine given in one course ranged from 1.6 to 8.4 gms., administered in from 2 to 10 days. The average dose was 4.6 gms., given in a 5 day period. These averages are a little higher than for the cases in the first group. Unpleasant symptoms were common. In the order of frequency these were: headache, palpitation, nausea or vomiting, epigastric distress, giddiness, diarrhea, precordial pain and fever. Tachycardia was the rule and in one case persisted until outspoken heart failure resulted. In this case the symptoms were promptly relieved by digitalis medication.

Two abnormal heart rhythms which give evidence of intoxication of the heart muscle by quinidine merit special consideration. The first of these is the *extrasystolic irregularity*, consisting of frequent ventricular premature contractions, sometimes coupled with normal beats. In quinidine as in digitalis therapy, this arrhythmia is an indication to desist from further exhibition of the drug. If the warning is not heeded, the second and more serious irregularity may follow. This is termed *ventricular tachycardia* and consists of a rapid volley of ectopic ventricular beats occurring at fairly regular intervals. Though this irregularity is readily detected with the electrocardiograph, it is difficult to diagnosticate with accuracy either by the ear or by means of polygraphic tracings (see Figure 1). As is evident from the case whose records are here reproduced, ventricular tachycardia may result from comparatively small doses of quinidine, *i.e.*, 1.2 gm. This arrhythmia assumes great clinical significance if it is borne in mind that in dogs injected with digitalis or strophanthin it is the immediate forerunner of ventricular fibrillation and death. Ventricular tachycardia has been observed five times in our series of cases, but has been promptly recognized and quinidine therapy at once discontinued. Under these circumstances it was transitory, disappearing in the course of several hours.

In the light of present knowledge, it is as yet difficult to select from the various cases of auricular fibrillation which present themselves for treatment those which will do well with quinidine. It is suggested

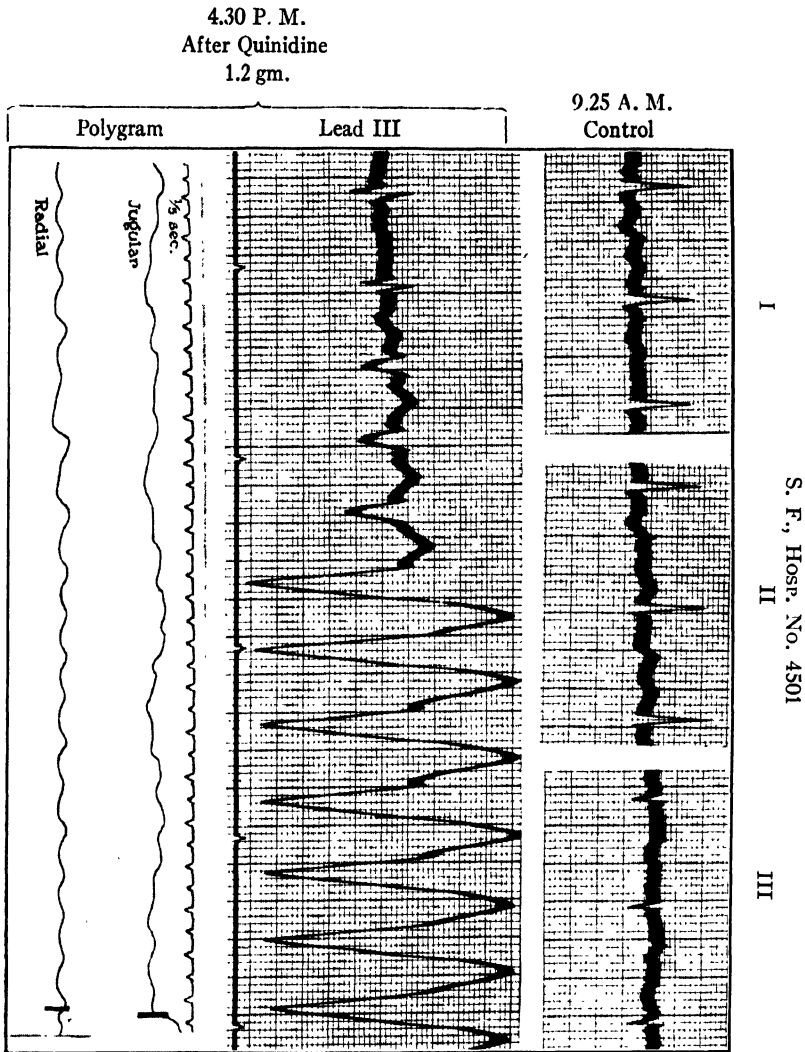


FIG. 1. 9.25 A. M.—Electrocardiogram (Leads I, II and III), shows auricular fibrillation. Quinidine 0.4 gm. given at 10.30 A. M., 12.30 and 2.30 P. M. 4.30 P. M.—Electrocardiogram (Lead III), shows a series of ectopic ventricular complexes followed by a rapid succession of ectopic beats having their origin in the left ventricle (ventricular tachycardia). The polygram, made at the same time, serves to illustrate the difficulty of diagnosing the arrhythmia by the use of jugular and radial tracings.

that patients with large hearts, especially if there are multiple valve lesions, be treated with caution. Cases without valvular disease and with but little evidence of hypertrophy have, in our relatively small series, fared well.

In view of the possibility of the occurrence of occasional embolic phenomena and of serious disturbances in the cardiac rhythm it must be urged that, until the criteria for choosing favorable cases are more clearly defined, patients with auricular fibrillation receive quinidine in bed, under careful supervision and preferably in a hospital, where the behavior of the heart can be studied with the aid of graphic records. Carefully administered, this drug is a therapeutic agent of great value; indiscriminately given, it may, on occasion, be expected to cause disastrous effects.

CLINICAL STUDIES OF QUINIDINE.

IV. THE CLINICAL TOXICOLOGY OF QUINIDINE.

By ROBERT L. LEVY, M.D.

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In the treatment of certain of the cardiac irregularities, particularly auricular fibrillation, quinidin sulphate is, at times, a useful agent in altering the cardiac mechanism.¹ A number of untoward effects have been noted in patients receiving this drug, and several fatalities have been ascribed to its use. It is of interest to inquire as to what has been the clinical experience with this alkaloid in the past, and it is important properly to evaluate the significance of the accidents that have more recently followed its administration.

Historical.

The alkaloid quinidin was first isolated by van Heijningen² in 1849 from a substance called chinoidin, a by-product in the preparation of quinin. He described it under the name "beta-chinin."

In 1853, Pasteur³ prepared the same drug, perhaps in somewhat purer form, and named it "chinidin." Hesse,⁴ in 1868, in referring to it, called it "conchinin;" other less used designations are "cinchotin" (Hlasiwetz), and "beta-chinidin" (Kerner). The sulphate is the form that has commonly been employed for therapeutic purposes.

¹ Levy, R. L.: Clinical Studies of Quinidin, I, Restoration of the Normal Cardiac Mechanism in Auricular Fibrillation by Quinidin, J. A. M. A. 76: 1289 (May 7) 1921; II, Alterations in the Cardiac Mechanism after Administration of Quinidin to Patients with Auricular Fibrillation, with Consideration of Certain Toxic Effects of the Drug, Arch. Int. Med., to be published; III, Some Results of the Quinidin Treatment of Auricular Fibrillation, New York State J. M. 22: 276 (June) 1922.

² Van Heijningen, J.: Ueber Chinoidin und B-Chinin, Pharm. Centralbl. 20: 465, 1849.

³ Pasteur, L.: Recherches sur les alcaloides des quinquinas, Compt. rend. Acad. d. sc. 36: 26, 1853.

⁴ Hesse, O.: Ueber Conchinin, Ann. d. Chem. u. Pharm. 146: 357, 1868.

In his original communication, van Heijningen makes casual mention of the fact that Bauduin, a Dutch clinician, found "beta-chinin" just as effective in the treatment of malaria as quinin. The first to make extensive use of quinidin in the clinic was Wunderlich,⁵ who gave it to patients with malaria. In the second edition of his *Handbuch* he says:

I have tried out the neutral sulphate of quinidin in more than 100 cases and have found it to be a preparation fully as efficacious as quinin, and at the present time give it almost exclusively in hospital practice because it is cheaper. In mild cases, 10 grains suffice; in more severe infections one may use 15 to 25 grains, seldom more, just as with quinin.

Why later Wunderlich abandoned the use of the drug is not clear. In the Leipzig clinic it was again extensively employed by Strümpell,⁶ who reported on its antipyretic action in fifty cases of infectious disease—malaria, typhoid, pneumonia, erysipelas, puerperal fever and pulmonary phthisis. The largest individual dose given was 1.5 gm. In typhoid fever, in addition to lowering of the temperature, there was observed slowing of the pulse, vomiting (occurring from one-quarter hour to three hours after taking), tinnitus aurium, sweating, faintness and, in one instance, collapse and death. This was in a woman of 32, who had received quinidin with good antipyretic effect on several occasions, but who, by mistake, swallowed 4 gm. in 60 cc. of water, the solution having been prepared for use as an enema. She vomited at once, and there were symptoms of collapse. Next day she had apparently recovered. Suddenly, however, and without ascertainable cause, she became unconscious, and heart and respiration temporarily ceased. She revived, but died seven days later without regaining consciousness.

At necropsy, there were found numerous deep typhoid ulcers in the intestine, and several small hemorrhages on the inner surface of the dura mater. The findings were otherwise negative. It is doubtful, Strümpell concludes, whether quinidin may be regarded as the cause

⁵ Wunderlich, C. A.: *Handbuch der Pathologie und Therapie*, Ed. 2, Stuttgart, 1856, p. 512.

⁶ Strümpell, A.: *Ueber die Anwendung und die antipyretische Wirkung des Conchinins*, Berl. klin. Wchnschr. 15: 679 (Nov. 18) 1878.

of death. But he warns against large doses (from 3 to 5 gm.), such as were evidently being used in other clinics.

Two years after these cases were described, Freudenberger⁷ recorded two sudden deaths in patients ill of typhoid, following the taking of comparatively small doses of quinidin. In one case, the severity of the disease may have been in large part responsible for the fatal issue. In both cases gastro-intestinal symptoms, i. e., nausea, vomiting and abdominal pain, were produced. He believed that death may have been due to violent vagus irritation and, reflexly, cessation of heart and respiration. As contraindications to the use of the drug, he cites cardiac weakness or signs of irritability in the domain of the vagus, as evidenced by vomiting, signs of peritoneal irritation, or severe gastric catarrh, such as occurs in chronic alcoholism.

Steffen,⁸ in 1882, reported two deaths in children, both of whom were suffering from scarlet fever. A girl, aged $8\frac{1}{2}$ years, received 1 gm. on the day of admission to the hospital, and two doses of 0.5 gm. each on the following day. One hour after the second dose, there were vomiting, collapse, convulsions and death in a few minutes. No necropsy was performed. The second patient, a girl, aged 4 years, received 9.5 gm. in seventeen days, usually not more than 1 gm. being given in a twenty-four hour period. One hour after the last dose, there were vomiting and convulsions, and thirty minutes later, death. Necropsy revealed inflammation of the pia mater, bronchitis and peri-bronchitis, inflammation and exudation in all the serous cavities, and inflammation of the kidneys. It is reasonable to question whether the last dose of the drug was entirely responsible for the fatal outcome in so sick a patient.

In the same communication is found a report of the occurrence of edema after quinidin. A boy, aged $7\frac{1}{2}$ years, with typhoid fever, received 12 gm. of quinidin in five days. He vomited several times, usually from one-half to one hour after taking the drug. On the fifth day, edema of the face and lower legs appeared, and on the sixth day there was general anasarca, with diarrhea. On the seventh day,

⁷ Freudenberger, J.: Ueber Conchinin und seine therapeutische Verwendung, *Deutsch. Arch. f. klin. Med.* 26: 577, 1880.

⁸ Steffen, A.: Ueber Conchinin und Hydrochinon, *Jahrb. f. Kinderh.* 18: 71, 1882.

diuresis, with a urinary output of 3,000 c.c., began, and continued on the eighth day, on which there was an output of 2,700 c.c. The transudates coincidentally disappeared.

Next day, because of the recurrence of fever, 2.5 gm. of quinidin was given. Again general anasarca resulted, with diuresis and disappearance of the edema on cessation of therapy. There was no dilatation of the heart, says Steffen, but the pulse was irregular and feeble.

The foregoing account of certain clinical experiences with quinidin during the period in which it was extensively employed in cases of malaria and as an antipyretic in the acute infectious diseases serves to indicate that even at that time undesirable effects were attributed to its use.

Untoward Effects of Quinidin in Auricular Fibrillation.

More than 460 cases of auricular fibrillation treated with quinidin have been reported during the last four years. Undoubtedly many more patients have received this drug. A variety of undesirable effects have been recorded; a number of accidents are, as I am well aware, not yet reported in the literature. The untoward effects may, for the purpose of discussion, be divided into six groups: (1) unpleasant symptoms attending administration; (2) induction of heart failure; (3) sudden collapse; (4) occurrence of heart rhythms indicating intoxication of the heart muscle; (5) embolism, and (6) sudden death.

1. *Unpleasant Symptoms.*—In our own experience, these have been relatively uncommon in the group of patients in whom normal rhythm was established (Table I). The dose in these cases has ranged from 0.4 to 8.4 gm., given in from one to seven days. The average dose was 3 gm. given over a three day period, according to a plan previously outlined.⁹ Two patients in this group who complained of discomfort are worthy of special mention. In both (Cases 3 and 24) the normal rhythm was of short duration, i.e., fourteen days and thirty-six hours, respectively. In Case 3, it was not possible on another occasion to restore the sinus rhythm. Case 24, discussed in more detail later, exhibited evidence of cerebral embolism. In short, these patients were unfavorable subjects for quinidin therapy.

⁹ Levy, R. L. (Footnote 1, third reference.)

In contrast to the paucity of complaints among this group, unpleasant symptoms were common in the cases in which it was not possible to restore the sinus rhythm. Headache and palpitation were the chief sources of discomfort. Next in frequency were nausea or vomiting, epigastric distress, giddiness, diarrhea, precordial pain, fever, tinnitus aurium, mental depression, excitement, flushing and sweating. Urticaria and scarlatiniform eruptions have also been observed, the latter notably by Ellis and Clark-Kennedy. Tachycardia was the rule. The dose ranged from 1.2 to 8.4 gm. given in from two to ten days. The average dose was 4.7 gm. given over a five day period. The average figures both for dose and for duration of administration are a little higher in this group and account perhaps in part, though certainly not entirely, for the discrepancy in the number of symptoms produced; for the complaints were usually made early in the course of therapy and, unless truly distressing, were disregarded in the hope that subsequently a change in cardiac mechanism might be produced.

2. *Induction of Heart Failure.*—In a previous communication,¹⁰ attention was directed to the possibility of the occurrence of heart failure as a result of the acceleration of ventricular rate caused by quinidin, and an illustrative case was reported in some detail. Another instance has been recorded by Eyster and Fahr.¹¹ By careful clinical observation, such an occurrence can be avoided; or, if it should transpire, the symptoms can be speedily alleviated by prompt and adequate digitalis therapy.

3. *Sudden Collapse.*—In one of his early papers, Frey,¹² who is responsible for pointing out the therapeutic possibilities of quinidin, cited two cases in which this accident occurred. Two more are reported by Haass,¹³ and a fifth by Frey and Hagemann.¹⁴ All recovered. Frey's description serves to illustrate the course of events.

¹⁰ Levy, R. L. (Footnote 1, second reference.)

¹¹ Eyster, J. A. E., and Fahr, G.: Observations on the Use of Quinidin in Auricular Fibrillation, *Arch. Int. Med.* **29**: 59 (Jan.) 1922.

¹² Frey, W.: Weitere Erfahrungen mit Chinidin bei absoluter Herzunregelmässigkeit, *Berl. klin. Wchnschr.* **55**: 849, 1918.

¹³ Haass, H.: Ueber die Chinidinterapie der unregelmässigen Herztätigkeit, *Berl. klin. Wchnschr.* **58**: 540 (May 23), 1921.

¹⁴ Frey, W., and Hagemann, E.: Klinische und experimentelle Daten über Chinidinwirkung, *Ztschr. f. d. ges. exper. Med.* **25**: 290, 1921.

TABLE I.
Symptoms Attending Quinidin Administration in Twenty-Five Cases of Auricular Fibrillation.

Case No.	Age, years.	Etiology.	Mitral disease.	Mitral and aortic disease.	No valve lesion.	Cardiac enlargement.	Quinidin therapy.		Symptoms.												Comment.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
							Total amount, gm.	Duration of admin., days.	Result.	Headache.	Tinnitus aurium.	Giddiness.	Palpitation.	Dyspnea.	Nausea or vomiting.	Epigastric distress.	Diarrhea.	Fever.	Precordial pain.	Mental symptoms.		Flushing.	Sweating.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
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Cases in which the normal rhythm was restored.

Ventricular tachycardia induced; duration of normal rhythm only 14 days.

Cerebral embolus 40 min. after onset of normal rhythm; died 2 weeks later with thrombosis of abdominal aorta.
Died suddenly 1 hour and 40 minutes after onset of normal rhythm.

432	Rheum.	+	++	4.8	5	0	+				Died 3 months later.
519	Rheum.	+	+++	4.2	7	0	+				Ventricular tachycardia induced.
623	Rheum.	+	+++	3.6	5	0	+				" "
758	Arterio-sclerosis	..	++++	3.2	5	0	+				" "
834	Rheum.	+	+++	6.2	8	0	+			+	
933	Rheum.	+	++	2.4	3	0	+		+		
1046	Syph. & Rheum.	+	++	3.0	4	0	+		+		Died 4 mos. later; at necropsy there was a thrombus in the left auricle, thrombosis of the pulmonary arteries and pulmonary infarction.
1155	Arterio-sclerosis.	..	+++	6.8	5	0	+		+		
1323	Rheum.	+	+++	4.4	5	0	+				
1537	Rheum.	..	++	8.4	10	0	+		+	+	
1648	Rheum.	+	+++	3.6	3	0	+		+		Ventricular tachycardia induced.
1946	Rheum.	+	++	6.0	4	0	+				
2030	Rheum.	+	++	7.6	5	0	+				" "
2323	Rheum.	+	+++	1.6	2	0	+		+	+	

Both of his patients received, on the first day, two doses of 0.4 gm. each; on the second day, five doses of 0.2 gm. About three hours after the last dose, symptoms occurred suggesting cerebral paralysis. The patients first complained of dizziness and of feeling hot. Shortly thereafter they sank suddenly into a state of deep unconsciousness. They became pale, the respirations were slowed and then stopped, and soon no pulse was palpable. Artificial respiration, epinephrin, camphor and caffein were resorted to, with resultant resumption of respiration and cardiac activity.



FIG. 1. (Case 24.) Drawing of the auricular aspect of the mitral valve.

In one case, consciousness returned in twenty minutes, but the patient remained in a peculiar state of mental excitement, having hallucinations and grimacing. There was great motor irritability as well. During this period, she understood all that was said to her. Three-quarters of an hour after the beginning of the attack, the patient was mentally clear and felt quite well.

In the second case, unconsciousness lasted for several hours, and on three occasions became dangerously deeper, as evidenced by cessation of breathing and enfeeblement of the heart's action. Beads of per-

spiration stood on the forehead, and the extremities felt cold. Then followed motor unrest, with shaking movements of the upper part of the body, such as are seen in quinin poisoning, according to Lewin.¹⁵ Four and a half hours after the beginning of the attack the patient became quieter, fell into a deep sleep, and awoke the following morning without the slightest knowledge of what had transpired. During

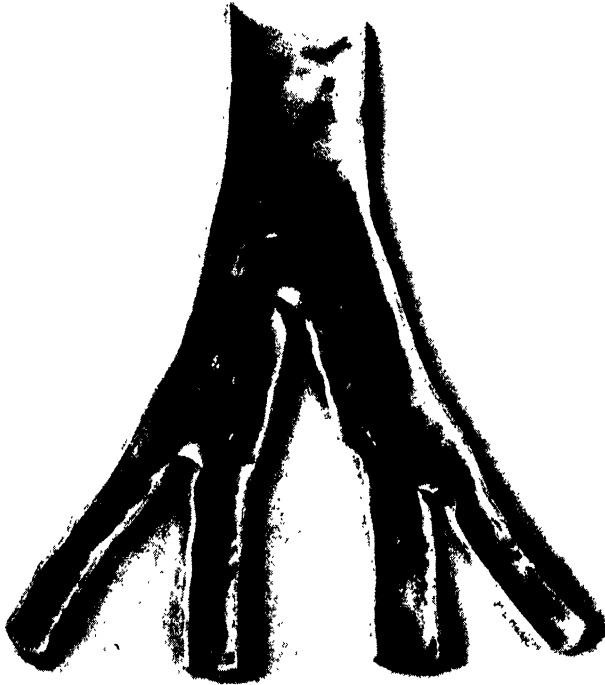


FIG. 2. (Case 24.) Drawing of the abdominal aorta dividing into its iliac branches, showing the occluding thrombus.

the entire next day the patient appeared to be psychically disturbed in that he was forgetful, and only on the day following was his condition the same as before the attack.

In Haass' patient, a woman, aged 53, with mitral stenosis and heart failure, collapse followed 2.8 gm. of quinidin given in three and one-

¹⁵ Lewin, L.: *Die Nebenwirkungen der Arzneimittel*, Berlin, 1899.

half days. She was revived with difficulty. However, her pulse subsequently became regular, and the signs of venous stasis disappeared. The happenings in the case of Frey and Hagemann were quite similar. A man, aged 37, received four doses of 0.2 gm. each. He complained of dizziness after the first dose. Shortly after the full amount was taken, unconsciousness ensued, to return after the lapse of one and one-half hours. He knew nothing of the onset of the attack or of the events which transpired during it.

There is nothing in the history or examination of these five patients which could serve to characterize them as peculiarly susceptible to the action of quinidin. It would be difficult to prognosticate and thereby avoid the possibility of an occasional occurrence of this nature.

4. *Occurrence of Rhythms Indicating Intoxication of the Heart Muscle.*—If the effects of quinidin administration are followed electrocardiographically, it is found¹⁰ that in a number of patients, particularly those in whom it is not possible to establish normal rhythm, ventricular premature beats appear. At times, though infrequently, these may occur coupled with normal complexes,¹⁰ as is seen in the early stage of digitalis poisoning. Occasionally such ectopic beats are transitory, disappearing within an hour or two. But often they persist and their frequency increases. Under the latter circumstances, they afford a sign indicating cessation of further therapy; for, if more quinidin is given, a succession of rapid, fairly regular ventricular ectopic beats may ensue, giving rise to the rhythm termed ventricular tachycardia. The possible serious significance of this disordered mechanism has already been indicated.¹⁶ Ventricular tachycardia may be the immediate precursor of ventricular fibrillation and death. This rhythm has been observed five times in our experience, but has ceased with prompt discontinuation of further drug treatment.

5. *Embolism.*—In Table II are collected the reported cases of embolism. Of these 9 patients, four died and one was left with a residual hemiplegia. The embolism occurred in seven instances within twenty-four hours after establishment of normal rhythm; in our own case, within forty minutes. Bits of clot lodged twice in the

¹⁶ Levy, R. L. (Footnote 1, second and third references.)

lung, three times in the brain, once in the kidneys, once in the spleen, once in both lung and kidney, and later, in this patient, *while the auricles were fibrillating*, in the popliteal artery. In only one of these cases was a necropsy performed: in Carter's case (Case 7, Table II). This patient died with the symptoms of uremia, and necropsy revealed a mural thrombus in the auricle and extensive infarction of both kidneys.

The patient whose history was detailed by me in an earlier communication⁹ subsequently died with the signs of closure of the abdominal aorta. A post-mortem examination was made. It will therefore be of interest briefly to recapitulate the sequence of events and to complete the record.

K. C., a woman, aged 44, was admitted to the hospital complaining of shortness of breath and weakness. She had frequent attacks of tonsillitis and three attacks of chorea between the ages of 6 and 14. Eight years before entrance, the tonsils were removed. She had had symptoms referable to the heart for two years, and it is probable that she was suffering from fibrillation of the auricles during this period.

Examination revealed no dyspnea at rest, and no edema or cyanosis. The cardiac dullness extended 12.5 cm. to the left in the fifth space, 4.5 cm. to the right in the fourth space. The auscultatory findings were those of mitral stenosis. She had been taking digitalis before coming to the hospital; the ventricular rate was 80. The blood pressure was 112 mm. of mercury systolic, and 82 diastolic. There was slight, irregular fever at times, up to 101 F.

She was kept in bed and given, in all, 0.5 gm. of digitalis in the form of digitan. On the tenth day of hospitalization, quinidin treatment was begun. During a six day period, 6.8 gm. was administered. The patient complained at various times of headache, tinnitus aurium and slight epigastric discomfort. There was slight acceleration of ventricular rate on each day. At 8 p. m. on the sixth day, normal rhythm was observed by the nurse to be present. At 8:40, quite suddenly the patient fell unconscious to the floor. After being put back to bed, she had a generalized convulsion, involving both arms and legs. She became very noisy, restless and difficult to manage. When examined at 9:30 she was still unconscious and quiet. There was no obvious sign of a local cerebral lesion. At 10:15 there was another convulsive seizure. By 1:30 the following morning, consciousness had returned. She vomited twice, and then slept during most of the remainder of the day.

Normal rhythm, with a rate of about 90, persisted for thirty-six hours. Then fibrillation recurred, with a ventricular rate of 126, 117 beats coming through to the wrist. Digitalis served promptly to control the heart rate.

TABLE II.

Reported Cases of Embolism after Quinidin.

Author and publication.	Embolism.		Result.	Comment.
	Site.	Time after establishment of normal rhythm.		
1. Benjamin and von Kapff: Deutsch. med. Wchnschr. 47: 10 (Jan. 6) 1921.	Lung.	12 hours	Death.	
2. Sappington: J. A. M. A. 78: 59 (Jan. 7) 1922.	Brain.	24 hours	"	
3. Frey and Hagemann: Ztschr. f. d. ges. exper. Med. 25: 290, 1921.	Lung.	2 hours	Recovery	
4. Ellis and Clark-Kennedy: Lancet 2: 894 (Oct. 29) 1921.	Lung, kidney; popliteal artery.	24 hours	"	
5. Ellis and Clark-Kennedy: Lancet 2: 894 (Oct. 29) 1921.	Spleen.	24 hours	"	Same case as above; embolism occurred before onset of normal rhythm.
6. Wilson and Herrmann: J. A. M. A. 78: 865 (March 25) 1922.	Brain.	8 hours	"	Residual palsy.
7. Carter, Dieuaide and Burwell: J. A. M. A. 78: 1921 (June 17) 1922.	Kidneys.	?	Death	Death from uremia.
8. Carter, Dieuaide and Burwell: J. A. M. A. 78: 1921 (June 17) 1922.		?	"	Details not given.
9. Levy: New York State J. Med. 22: 276 (June) 1922.	Brain.	40 minutes	Recovery	Death 3 weeks later with thrombosis of abdominal aorta.

During the next twelve days, the clinical course was uneventful, save for an elevation of temperature to 102 F. on two occasions. Blood cultures were sterile. On the morning of the thirteenth day after the cerebral accident, the patient was awakened at 4:30 by severe pain in the left side of the abdomen. At this time she also complained that her feet, legs and hips were quite numb. She presented the appearance of a person in shock. The heart rate rose rapidly to 144; the temperature was subnormal. There was some tenderness in the abdomen in the median line over the abdominal aorta, and also in the left lower quadrant. No pulsation could be made out in either femoral vessel, or in the popliteal or anterior tibial arteries. There was a little purplish mottling of the feet and lower legs. A diagnosis of thrombotic closure of the abdominal aorta was made.

The following morning, the mottled cyanosis extended to the umbilicus. The patient was restless, pale and perspiring freely. A distinct pulsation could now be felt in the right femoral artery, and a less distinct wave in the left femoral vessel. The mottled cyanosis of the feet, legs and thighs was more pronounced. That afternoon, she died.

Necropsy was performed four hours after death. The heart weighed 430 gm. The mitral valve was deformed and thickened in such a fashion as to produce an orifice of the buttonhole variety. The chordae tendineae was contracted and thickened. There were no fresh vegetations along the free border of the valve cusps. On the auricular aspect of the valve, however, adherent to the wall of the auricle, at a point between the valve cusps, was a small, pear-shaped vegetation about 3 by 2 mm., its surface covered by a thin layer of adherent clot. The wall of the auricle was smooth, and there were no antemortem thrombi (Fig. 1). The aortic valve cusps were smooth. On the tricuspid valve were two tiny nodular vegetations, of the variety commonly associated with acute vegetative endocarditis; these were about 1 mm. in diameter. The heart muscle was not remarkable.

The lungs showed no areas of consolidation or infarction. The pulmonary vessels were dissected out to their finer branches, but no thrombi or emboli were disclosed.

When the aorta and iliac vessels were opened, an adherent, fresh, occluding thrombus was seen which had apparently begun at the bifurcation of the aorta into the iliac branches and extended upward, though not filling the entire lumen of the vessel, as far as the renal arteries. The thrombus also extended downward for a distance of 3 or 4 cm. into both external and internal iliac arteries (Fig. 2). No thrombi were found in the vena cava or its branches, nor was there any evidence of occlusion of any of the mesenteric vessels.

Permission to examine the brain was not granted. In view of this fact and in the light of the necropsy findings it is perhaps not justifiable to place too categorical an interpretation on the cerebral accident. It is conceivable that the attack of unconsciousness with convulsions may have been a manifestation of quinidin poisoning analogous to the

cases described by Frey. On the other hand, the association of the attack with the establishment of normal rhythm is significant. It seems more likely that a small bit of the vegetation on the auricular aspect of the mitral valve was whipped off into the circulation and lodged in the brain. This conception is supported by later events; for it is reasonable to believe that the terminal thrombosis in the aorta was initiated by a small embolus from the same source, which became caught at the bifurcation of the aorta into its iliac branches and served as a nucleus for the formation of the larger clot.

It is a matter of common belief that clots tend to form in the auricular appendixes more commonly when the auricles are fibrillating than when the mechanism is normal. The findings of Lewis¹⁷ lend support to this concept. Thus, in seventy-six post-mortems in cases of chronic heart disease, in which clots were especially sought, he found them in eight cases out of twenty-three in which fibrillation was present in the last illness, and in only four cases out of fifty-three in which the mechanism had been normal. It was his impression, however, that embolism due to detachment of these clots did not appear to be more common when fibrillation existed than when the mechanism was normal. This statement is borne out in our own experience. Of the last fifty patients with auricular fibrillation admitted to the Rockefeller Hospital, twenty-five received the usual therapy, with digitalis if indicated; twenty-five received similar treatment, plus quinidin. In the group not treated with quinidin, embolism or thrombosis has occurred five times; in the group receiving quinidin, there has been one instance of probable embolism. It is, of course, significant that in the patients receiving quinidin, embolism has occurred shortly after the alteration in mechanism. Mackenzie¹⁸ has called attention to a similar coincidence when the rhythm spontaneously changes from fibrillation to normal. It appears, however, that the danger of embolic accident after quinidin has been given undue emphasis. It is in patients with advanced heart failure that clots are likely to form in the auricles. If such cases do not respond promptly

¹⁷ Lewis, T.: The Value of Quinidin in Cases of Auricular Fibrillation, and Methods of Studying the Clinical Reaction, *Am. J. M. Sc.* 163: 781, 1922.

¹⁸ Mackenzie, J.: Quinidin in Auricular Fibrillation (a letter), *Brit. M. J.* 2: 576 (Oct. 8) 1921.

and well to efforts at treatment aimed to build up a cardiac reserve, especially if they are refractory to digitalis, it is probably wiser not to attempt to restore the normal mechanism. Signs of peripheral venous thrombosis should also be looked for, and, if present, serve as an index of a tendency for intravascular clots to form. In such individuals also, it is well not to attempt quinidin therapy.

6. *Sudden Death*.—There are three cases reported in which death followed shortly after the resumption of normal rhythm. Instances in which, at necropsy, bronchopneumonia was found (Benjamin and von Kapff) or in which death occurred several days after quinidin was taken (Hewlett and Sweeney), are not included. Nor is it fair to place in this group Groedel's patient, a man of 70, with heart block and Stokes-Adams' syndrome, whose heart at necropsy showed closure of the septal branch of the right coronary artery, with myomalacia and complete destruction of the conduction system in the crus commune.¹⁹ In the case of Benjamin and von Kapff,²⁰ 0.2 gm. of the drug was given on the first day, and 0.6 gm. on the following day. Forty-five minutes after the establishment of normal rhythm, the heart suddenly ceased. Hewlett and Sweeney's case is described in a footnote: "A patient in fairly good compensation was treated with quinidin and died suddenly shortly after his heart had become regular."²¹ No necropsy was performed in either of these cases. One instance of sudden death has occurred in this hospital and has been described in detail.⁹ The heart ceased one hour and forty minutes after inception of the sinus rhythm. Necropsy failed to reveal the cause of death. In the absence of facts, speculation as to the reason for the cessation of the heart's action under these circumstances is futile.

Comment and Conclusions.

From this summary of the untoward effects of quinidin administration, several points of significance for clinical therapeutics are apparent.

¹⁹ Groedel, F. M.: Ungünstige Chinidinwirkung bei vollkommenem Herzblock? Therap. d. Gegenw. 62: 172, 1921.

²⁰ Benjamin and von Kapff: Ueber die Behandlung der Arrhythmia perpetua mit Chinidin, Deutsch. med. Wchnschr. 47: 10 (Jan. 6) 1921.

²¹ Hewlett, A. W., and Sweeney, J. P.: The Quinidin Treatment of Auricular Fibrillation, J. A. M. A. 77: 1793 (Dec. 3) 1921.

It is clear that not every patient suffering from fibrillation of the auricles should receive this drug. It has already been pointed out,⁹ and it is evident from Table I, that persons with conspicuous cardiac enlargement, that is, with apex impulse outside the nipple line, and particularly if there are multiple valve lesions, are least likely to respond with normal rhythm. Such enlargement may be taken to indicate serious derangement of cardiac mechanics by valvular disease or of extensive myocardial involvement, or both. It is in these cases also that ventricular tachycardia occasionally occurs. Patients with but little cardiac enlargement and without signs of valvular defect have, in our limited experience, done well. The duration of fibrillation and the etiology of the disease appear to be of lesser importance as factors determining the issue.²²

A careful study of the patient's symptoms while the drug is being given may also be of value. When unpleasant sensations are complained of early in the course of treatment, and persist or recur with further administration, it is probable that normal rhythm will not be restored, or, if established, will be present for only a short time. It is in these cases also that ventricular tachycardia is likely to ensue. The occurrence of unpleasant symptoms may likewise serve as a guide by means of which cases of sudden collapse, as described by Frey, may be avoided. At all events, little is to be gained and possibly harm may be done, by persisting with therapy in the face of complaint of serious discomfort by the patient.

Occasionally one encounters a patient in whom, in spite of rest, restricted fluid intake, digitalis, etc., it is not possible to restore adequate compensation. The heart muscle is exhausted. It is in such cases that mural thrombi are likely to form in the auricles, and it is in them that the danger of embolism after alteration of the cardiac mechanism is greatest. Signs of peripheral venous thrombosis should also be looked for, and, if found, serve to indicate the tendency for the formation of intravascular clots. Such patients should not be given quinidin.

Careful clinical study of patients with auricular fibrillation will serve to set apart a certain number unsuitable for quinidin treatment. It

²² Experience with auricular fibrillation in patients with hyperthyroidism is as yet too limited to permit of defining helpful criteria in this group.

is hoped that further experience will serve to furnish more definite criteria for eliminating unfavorable and selecting favorable cases. The carrying out of therapy in bed, in a hospital, preferably under graphic control, will further safeguard against accident. It is well to emphasize again that, "carefully administered, this drug is a therapeutic agent of great value; indiscriminately given, it may, on occasion, be expected to cause disastrous results."

EXPERIMENTAL STUDIES IN DIABETES.

The following articles on diabetes have appeared in *The Journal of Metabolic Research*. As the work is so extensive it has been considered advisable not to reprint the papers in the *Studies*.

- (a) WISHART, MARY B. Experimental studies in diabetes. Series IV. Lipemia. 1. Analyses of blood lipoids in diabetic animals and patients. *J. Metabol. Research*, August, 1922, ii, pp. 199-217.
- (b) ALLEN, FREDERICK M. Experimental studies in diabetes. Series IV. Lipemia. 2. The production of diabetic lipemia in animals, and observations on some possible etiologic factors. *J. Metabol. Research*, August, 1922, ii, pp. 219-298.

THE HYDROGEN ION CONCENTRATION OF JOINT EXUDATES IN RHEUMATIC FEVER AND OTHER FORMS OF ARTHRITIS.

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(Received for publication, May 19, 1922.)

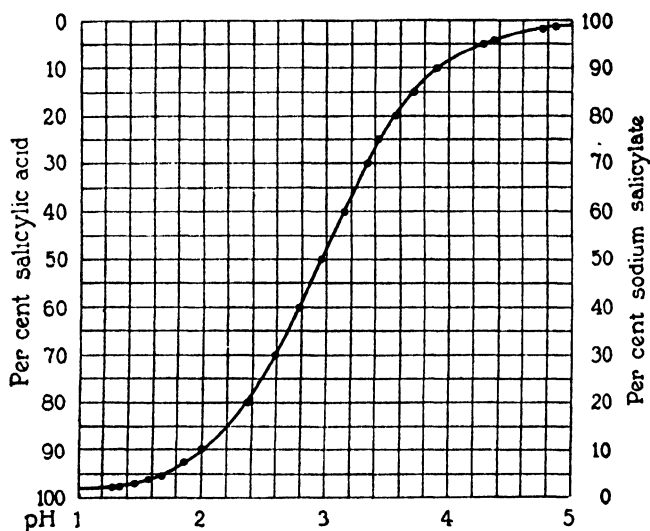
In connection with a study of acute rheumatic fever we have determined the hydrogen ion concentration of exudates aspirated from the inflamed joints of patients ill with this disease and also of exudates of patients with certain other forms of arthritis. This was done (1) to compare the reactions of the exudates in these arthritic diseases, and (2) to determine whether an acidity occurs in the inflamed joints in acute rheumatic fever sufficient to permit the liberation of free salicylic acid following salicylate therapy.

One explanation of the action of salicylates in patients with rheumatic fever has been based upon the hypothesis that free salicylic acid is liberated in the inflamed joint. If the inflammation in the joint results from the local irritation of bacteria, a bactericidal action of free salicylic acid might explain the improvement that ordinarily follows the administration of this drug. This theory originated with Binz (1), who realized that free salicylic acid could not exist in normal blood and tissues, and also that the salts of salicylates are not bactericidal in weak solutions. In subjecting sodium salicylate solutions to high CO₂ tensions *in vitro*, he found that sufficient acid was liberated to be bactericidal. The reason for the liberation of free salicylic acid in Binz' experiments is obvious: the increased CO₂ tension caused sufficient acidity to allow dissociation of the acid. He thought that a similar increased CO₂ tension occurred in the inflamed tissues in acute rheumatic fever because Ewald (2) had previously shown that such an increase could occur in certain types of inflammatory exudates. Although Binz' theory seemed unsatisfactory, it has never been conclusively disproven and is quoted in most pharmacologies.

Hanzlik and his collaborators (3), in an extensive investigation of the pharmacology of salicylates, examined joint fluids directly by the ferric alum test for the presence of free salicylic acid; the patients with rheumatic fever received full therapeutic doses of the drug. These authors found no free salicylic acid present. A criticism of this work was made by the authors themselves who state that: "Objection might be raised to the method used because of the possibility that

some CO_2 is lost when the fluid is exposed to the atmosphere, but this is to a considerable extent prevented by the presence of 'buffer' or protective substances."

Reaction at Which Free Salicylate Acid Occurs.—Hanzlik (4) also found that salicylic acid was released from mixtures of sodium salicylate and buffer salts only when the acidity was greater than pH 6.7. The iron test for salicylic acid was found to be faintly positive at a pH of 6.7 and progressively stronger with an increase in acidity from pH 6.5 to 1.0; on the alkaline side of pH 6.7 no salicylic acid was liberated. Mixtures containing 25 per cent serum



TEXT-FIG. 1. Salicylic acid-sodium salicylate curve at 40°C.

or plasma did not show any free salicylic acid between pH 7.4 and 5.9; tests were not carried out at a higher acidity. He states that: "It is conceivable that very low degrees of acidity, i.e., in the neighborhood of pH = 6.8 or 6.9, might occur in closed cavities with sluggish circulation as in the articulations," but "fluids of joints and similarly enclosed regions would need to be more highly acid and freer from protein and other constituents than is probably the case in order to contain free salicylic acid and explain the therapeutic relief from salicylate medication according to the antiseptic theory."

The dissociation curve for mixtures of salicylic acid and sodium salicylate is given in Text-fig. 1. It is evident that at reactions more

alkaline than pH 5.0 the free acid constitutes less than 1 per cent or the total salicylate. Since it has been estimated that following full therapeutic doses of salicylate the concentration in the blood or joint fluid is about 0.02 per cent (3), the amount of free acid present at a pH of 6.0 would be less than 0.0002 per cent; *i.e.*, an amount that could exert no bactericidal action. This conclusion is in agreement with Hanzlik's experimental findings.

EXPERIMENTAL.

The reactions of twenty-six joint exudates have been determined. The majority of these were from patients ill with acute rheumatic fever; a number were from patients having arthritis of unknown origin (chronic arthritis, intermittent hydrarthrosis); two were from patients with definite bacterial arthritis; and one, a simple effusion, was from a patient with myocardial insufficiency and generalized edema.

In each instance the joint was aspirated with a tightly fitting Luer syringe containing a small amount of sterile paraffin oil to prevent the admission of any air bubbles. During the entire determination the fluid was prevented from coming in contact with the air.

Owing to the fact that a considerable quantity of fluid was necessary for electrometric pH determinations and that in each instance several additional cubic centimeters were needed for culture, colorimetric determinations were made in the majority of instances. When large quantities of fluid were available, both colorimetric and electrometric determinations were made, in order to obtain the factor necessary to convert colorimetric readings at room temperature to electrometric at body temperature (38°C.).

*Electrometric Measurements.*¹—The electrometric determinations were made at 38°C. in the Clark cell, in a hydrogen atmosphere containing CO₂ at the tension existing in the joint fluid. The determinations were corrected for partial pressures of CO₂ and H₂O to one atmosphere of dry H₂. The solution used in the standardization of the hydrogen electrode was 0.1 N HCl whose pH at 38° was assumed to be 1.09. This brings the pH determinations to the basis of Sören-

¹ A number of these determinations were made by Dr. A. B. Hastings.

sen's standards. A detailed description of the technique employed has been recently published by Cullen (5).

Colorimetric Measurements.—The method was that described by Cullen (5) for the colorimetric determination of the hydrogen ion concentration of blood. 1 cc. of the aspirated joint fluid (usually within 4 minutes of withdrawal and before coagulation occurred) was added to a standard tube containing 20 cc. of 0.9 per cent sodium chloride solution and 7 drops of indicator; the addition was made under paraffin oil to prevent the escape of any CO_2 . 1 cc. of the same joint fluid was added to another tube containing 20 cc. of saline solution without any indicator to serve as a turbidity control in the comparator. The pH determination was then made by placing the tube in a comparator block and comparing it with standard color tubes. This was done at room temperature. Phenol red was used as indicator for determinations above pH 6.8 and brom-cresol purple for those below pH 6.8. All glassware, mineral oil, and salt solution were previously tested for neutrality. As standards, Sørensen's phosphate standards were prepared in steps of pH 0.2 from pH 5.6 to 6.8 and in steps of pH 0.05 from pH of 6.8 to 8.0. To those above pH 6.8, 5 drops of 0.03 per cent phenol red solution were added to each 15 cc. of standard; to those below pH 6.8 a similar quantity of brom-cresol purple was added.

The colorimetric determination at room temperature was corrected to 38°C. by the following formula:

$$\text{pH}_{38} = \text{colorimetric pH} + 0.01 (t - 20^\circ) \text{ pH} - 0.21 \text{ pH}$$

in which t = room temperature and 0.21 pH represents the empirical correction for temperature ($38^\circ - 20^\circ$), dilution, and protein errors. This correction represents the average of the differences between electrometric determinations at 38°C. undiluted, and the colorimetric determinations, diluted, at 20°C.; it corresponds closely with the correction similarly found for correcting colorimetric determinations of human blood. The room temperature in these experiments varied within $\pm 3^\circ\text{C}$. from 20°C. and a temperature change of 0.01 pH was made for each degree of variation.

Physical Characteristics of the Exudates Examined.—The fluids aspirated from the inflamed joints of patients ill with acute rheumatic

fever were never frankly purulent. They were viscous, slightly to distinctly turbid, and usually of a pale yellowish green color. Sometimes when the exudate was aspirated from a joint during the early stage of the arthritis, the greenish yellow shade was absent and the fluid had a grayish turbidity; this effect was probably due to a higher leucocyte content. The exudates contained considerable fibrin and formed soft clots on standing. Bacteriologically, they were sterile by ordinary culture methods. The fluids from the patients with arthritis of undetermined origin were indistinguishable from some of these exudates. The joint fluid from the patient with myocardial insufficiency was less turbid, did not clot so quickly, and had a lower leucocyte count than most of the rheumatic fever exudates. The exudates of the two patients with bacterial arthritis were easily distinguished as coming from infected joints. They were purulent fluids and contained bacteria.

Results.

Hydrogen Ion Concentration.—The results are shown in Table I. With the exception of the joint exudates of the two patients with bacterial arthritis, the reactions were all slightly alkaline and approximated the normal reaction of blood. The hydrogen ion determinations of sixteen fluids from patients with acute rheumatic fever varied between pH 7.27 and 7.42. Seven fluids were examined from three patients with arthritis of undetermined origin; one of these was diagnosed as intermittent hydrarthrosis, one as chronic arthritis, and the third was probably arthritis accompanying serum disease. Their reactions were approximately the same as those in acute rheumatic fever, varying between pH 7.33 and 7.47. The joint fluids of the two patients with bacterial arthritis were both definitely acid; the exudate from the knee joint infected with *Staphylococcus aureus* was pH 6.69; from the one with *Streptococcus hæmolyticus*, pH 6.19. The electro-metric determination of this latter fluid cannot be considered accurate as the fluid was kept over night in the ice box and heated to 56°C. for 1 hour to kill the organisms; the result, however, is sufficiently accurate to show the fluid to be definitely acid. The simple effusion from the patient with myocardial insufficiency accompanied by general edema was pH 7.34.

TABLE I.

Hydrogen Ion Concentration of Joint Exudates.

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations.			
						Colorimetric at 20°C.	Electrometric at 38°C.	Difference between colorimetric at 20°C. and electrometric at 38°C.	Colorimetric calculated at 38°C.†

Acute rheumatic fever.									
	*F.		days			pH	pH	pH	pH
4444	104.5	Right knee.	3	+++	No growth.	7.65	7.39	0.26	7.39
4350	102	Left wrist.	5	+	" "	7.53‡			7.32
4453	102	Right knee.	1	+++	" "	7.58	7.34	0.24	7.34
	99	Left "	7	+	" "	7.58			7.37
4367	100.5	Right "	10	+	" "	7.63‡			7.42
	104	Left "	2	++	" "	7.56‡			7.35
	104	Right "	2	++	" "	7.57‡			7.36
4417	103	" "	8	+	" "	7.54‡			7.33
	103	Left "	3	++	" "	7.54‡			7.33
	102.5	" "	5	+	" "	7.55‡			7.34
	102.5	Right "	12	+	" "	7.57‡			7.36
4473	103	Left "	1	+++	" "	7.49	7.27	0.22	7.27
	103	" "	2	++	" "	7.60			7.39
4485	103.8	Right "	1	+++	" "	7.54			7.33
	103.8	Left "	3	++	" "	7.56			7.35
4493	103	" "	4	+++	" "	7.51	7.34	0.17	7.34

* + indicates swelling only; ++, moderate inflammation; +++, marked inflammation.

† Average correction of pH 0.21 used except where actual difference is given.

‡ Room temperature not recorded. Variation not more than $\pm 3^{\circ}\text{C}.$ from $20^{\circ}\text{C}.$

TABLE I—*Concluded.*

Case No.	Patient's temperature at time of aspiration.	Joint.	Length of time joint aspirated was involved.	Degree of inflammation.*	Bacteriological examination.	Determinations.			
						Colorimetric at 20°C.	Electrometric at 35°C.	Difference between colorimetric at 20°C. and electrometric at 35°C.	Colorimetric calculated at 35°C.†
Arthritis of undetermined origin (includes chronic arthritis and intermittent hydrarthrosis).									
4451	101	Right knee.	2	+++	No growth.	7.54			7.33
	102	Left "	5	+++	" "	7.65			7.44
4428	99	Right "	?	+	" "	7.61	7.43	0.18	7.43
	100	" "	2	+	" "	7.67	7.42	0.25	7.42
	99	" "	2	+	" "	7.62	7.42	0.20	7.42
	98.5	" "	30 mos.	+	" "	7.68			7.47
4449	99	Left "	5	+	" "	7.60	7.42	0.18	7.42
Bacterial arthritis.									
4262	104	Right knee.	8	+++	<i>Staphylococcus aureus.</i>	6.90			6.69
4467	104.2	Left "	?	+++	<i>Streptococcus hemolyticus.</i>	6.40§ 6.10	5.89	0.21	6.19
Joint effusion, myocardial insufficiency.									
4458	102	Left knee.	?	+	No growth.	7.55			7.34

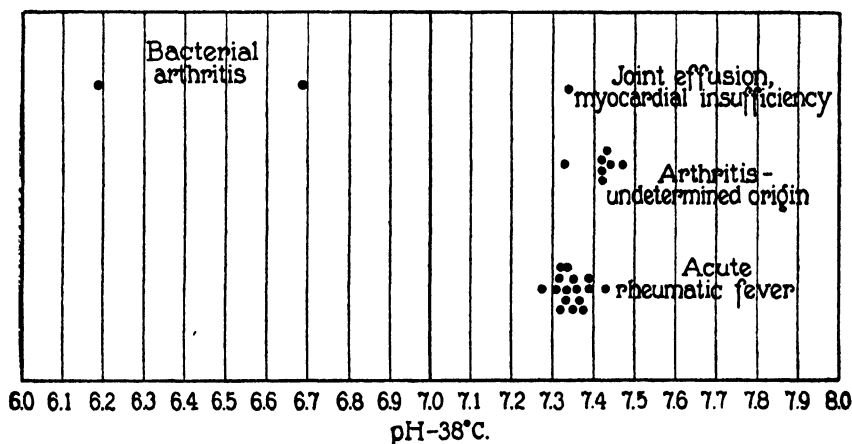
§ Within 4 minutes of aspiration.

|| Determined after standing in ice box over night and heated at 56°C. for 1 hour to kill bacteria.

DISCUSSION.

A comparison of the results is indicated in Text-fig. 2. It shows that the joint exudates on the basis of their reactions fall into two groups; the one having a slightly acid, the other a slightly alkaline reaction. The fluids containing bacteria fell into the first, the sterile fluids into the second. In general, these findings correspond with those of other investigators who have recently made hydrogen ion

determinations on various types of exudates. Shearer and Parsons (6) found the purulent spinal fluid of epidemic cerebrospinal meningitis to be about pH 6.9. Lord (7) found the pH of pneumonic lung exudates to be as low as 5.4. Shade and his coworkers (8) determined the reactions of many exudates electrometrically; pus from acute infections varied in pH between 5.96 and 6.57; pus from chronic inflammations such as tuberculosis between 6.58 and 7.00; serous exudates as in tuberculous pleurisy between 7.00 and 7.09; and non-inflammatory transudates between pH 7.17 and 7.24.



TEXT-FIG. 2. Hydrogen ion concentration of joint exudates.

It seems to us that only in the instance of purulent exudates would the liberation of free salicylic acid be at all possible. If acid is liberated in these exudates it is not sufficient to be bactericidal, as clinical observations have never shown any improvement in purulent arthritis following salicylate treatment. As Hanzlik has previously concluded from direct examinations that free salicylic acid could not be demonstrated in the joint exudates of patients with acute rheumatic fever, and our findings indicate that free acid could not possibly exist, it is evident that the local antiphlogistic effect of the drug is due to some other factor than free salicylic acid.

The determination of the reactions of these fluids has thrown some light upon the nature of the pathological process in the arthritis of

acute rheumatic fever. The results of various workers already mentioned show that when bacteria such as hemolytic streptococci, staphylococci, pneumococci, meningococci, or tubercle bacilli are present in an exudate in sufficient numbers to be detected, either by cultural or microscopic examination, the reaction of that exudate is always more acid than blood. We have evidence to show that the exudates in experimental arthritis of animals inoculated with green streptococci are also acid. From these results it would seem that if green streptococci were growing in the joint fluids of rheumatic fever patients, one should expect those fluids to be acid. But our examinations have shown that this is not the case. It is highly improbable, therefore, that one could cultivate streptococci from such exudates. This is in agreement with our bacteriological studies.

Another possibility must be considered: the etiologic agent—whatever it may be—may exist only in the capsule or contiguous tissues; and the exudate may be similar in nature to the serofibrinous fluid often found in pleurisy secondary to pneumonia. In many instances these pleuritic fluids are sterile and the patients recover without developing empyema. While there are not sufficient data concerning the reaction of such fluids, from the report of Shade we would expect them to be more alkaline than pH 7.0. Further observations should be made concerning the reaction of sterile fluids in serous cavities contiguous to foci of inflammation resulting from bacterial infection. When this information is available we shall be in a better position to evaluate properly the relation of our findings to the etiology and pathology of rheumatic fever.

The slight variation in the reactions of the exudates of acute rheumatic fever was not dependent upon the severity of the inflammation as evidenced clinically. The exudates from acutely inflamed joints were of about the same pH as those from joints in which the clinical signs of acuteness had disappeared and the only remaining evidence of involvement was the presence of fluid. The most acid exudate, having a pH 7.27, was found in a highly inflamed joint; the most alkaline fluid, having a pH 7.42, was found in a joint from which all acute signs of inflammation had disappeared; one highly inflamed joint, however, had a pH of 7.39 (Table II).

TABLE II.

Acute Rheumatic Fever.

Comparison of Exudates from Acutely Inflamed Joints with Those in Which Signs of Acute Inflammation Had Subsided.

Exudates from joints acutely inflamed.....	pH ₂₅ = 7.27—7.39
“ “ “ in which inflammation had subsided.....	pH ₂₅ = 7.31—7.42

SUMMARY AND CONCLUSIONS.

1. The hydrogen ion concentration of joint exudates aspirated from patients ill with acute rheumatic fever, arthritis of undetermined origin, and bacterial arthritis was determined. The hydrogen ion concentrations of the joint exudates from patients with acute rheumatic fever approximated the normal reaction of blood, varying from pH 7.27 to 7.42. Exudates from patients with arthritis of undetermined origin varied in pH from 7.33 to 7.47. The pH of a joint effusion occurring in a patient with myocardial insufficiency was 7.34. Bacteriologically, all of these fluids were sterile by ordinary means of cultivation. An exudate aspirated from a knee infected with *Staphylococcus aureus* had a pH of 6.69, while that from a patient having an arthritis due to *Streptococcus hemolyticus* was also acid, having a pH of 6.19.

2. Since a definitely acid medium is necessary for the liberation of free salicylic acid and since all of the joint fluids from patients with acute rheumatic fever were slightly alkaline, no free salicylic acid could possibly exist in such joint fluids following the administration of salicylates.

BIBLIOGRAPHY.

1. Binz, C., *Berl. klin. Woch.*, 1876, xxvii, 385; *Arch. exp. Path. u. Pharmacol.*, 1879, x, 147.
2. Ewald, C. A., *Arch. Anat., Physiol. u. wissenschaft. Med.*, 1876, 422.
3. Scott, R. W., Thoburn, T. W., and Hanzlik, P. J., *J. Pharmacol. and Exp. Therap.*, 1916-17, ix, 217.
4. Hanzlik, P. J., *J. Pharmacol. and Exp. Therap.*, 1921, xvii, 385.
5. Cullen, G. E., *J. Biol. Chem.*, 1922, lli, 501.
6. Shearer, C., and Parsons, T. R., *Quart. J. Med.*, 1921, xiv, 120.
7. Lord, F. T., *J. Am. Med. Assn.*, 1919, lxxii, 1364.
8. Shade, H., Neukirch, P., and Halpert, H., *Z. ges. exp. Med.*, 1921, xxiv, 11.

THE FOOD REQUIREMENTS OF CHILDREN.

V. PERCENTAGE DISTRIBUTION OF CALORIES.

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In the preceding articles in this series we have discussed the total caloric need of the average healthy child and the amounts of the different food constituents—protein, fat and carbohydrate—which should make up the diet. The next point to be considered is the relation of these food constituents to each other and the relation of each to the diet as a whole; in other words, the percentage distribution of the total calories into those given as fat, as protein and as carbohydrate.

In order to determine the usual relation of the constituents of the diet, we have calculated the percentage distribution of the calories in the diets of the 106 healthy children from 1 to 18 years of age studied by us. The values thus obtained for the individual observations are shown in Chart 1. The proportion of the total calories taken as protein is shown at the bottom, that as fat in the middle and that as carbohydrate at the top.

It is evident that the proportion of the calories taken as protein shows the least variation. The average value for all the cases, regardless of age, is 15 per cent. The range is from 11 to 23 per cent., while more than one-half the values are between 14 and 16 per cent.

The per cent. of the total calories taken as fat varies much more widely. The range is from 21 to 51 per cent., but three-fourths of the values are between 30 and 40 per cent. The average of all the cases is 34 per cent.

The proportion of the total calories taken as carbohydrate also shows wide variation. The range is from 38 to 65 per cent., but three-fourths of the values are between 44 and 55 per cent. The average is 51 per cent.

There was no regular variation with age, although the per cent. of the total calories taken as protein was slightly higher in the early

years. This is more clearly brought out in Table I, which gives the average distribution of the calories according to years.

Although there are fairly wide individual variations, it is evident that in the mixed diet commonly taken by healthy children the calories are distributed with surprising regularity. The average of the values obtained in our cases may be assumed to represent the usual distribution of the calories in the diet of healthy children. It

TABLE I.

Average Percentage Distribution of Calories Taken by Children.

Age, years.	No. of cases.	Fat.	Carbohydrate.	Protein.
1-2.....	7	33	50	17
2-3.....	11	33	51	16
3-4.....	10	34	49	17
4-5.....	12	36	48	16
5-6.....	10	33	52	15
6-7.....	8	35	51	14
7-8.....	5	35	52	13
8-9.....	9	34	52	14
9-10.....	9	34	52	14
10-11.....	9	32	52	16
11-12.....	4	38	48	14
12-13.....	3	37	50	13
13-14.....	1	34	53	13
14-15.....	3	32	54	14
15-16.....	3	33	53	14
16-17.....	1	36	50	14
17-18.....	1	37	44	19
Total.....	106			
Average.....	...	34	51	15

seems reasonable to make this assumption, when the large number of cases studied is considered, and when it is recalled that the children observed were not a group under any one supervision, but were children in private families in rather scattered localities. Not all of the children lived in New York City. Several lived in Boston, two in Pittsburgh, one in the South and many in New Jersey and Long Island. Regardless of age, the average distribution of the calories taken was as follows; fat, 34 per cent.; carbohydrate, 51 per cent.; protein, 15 per cent. This may conveniently be expressed in round

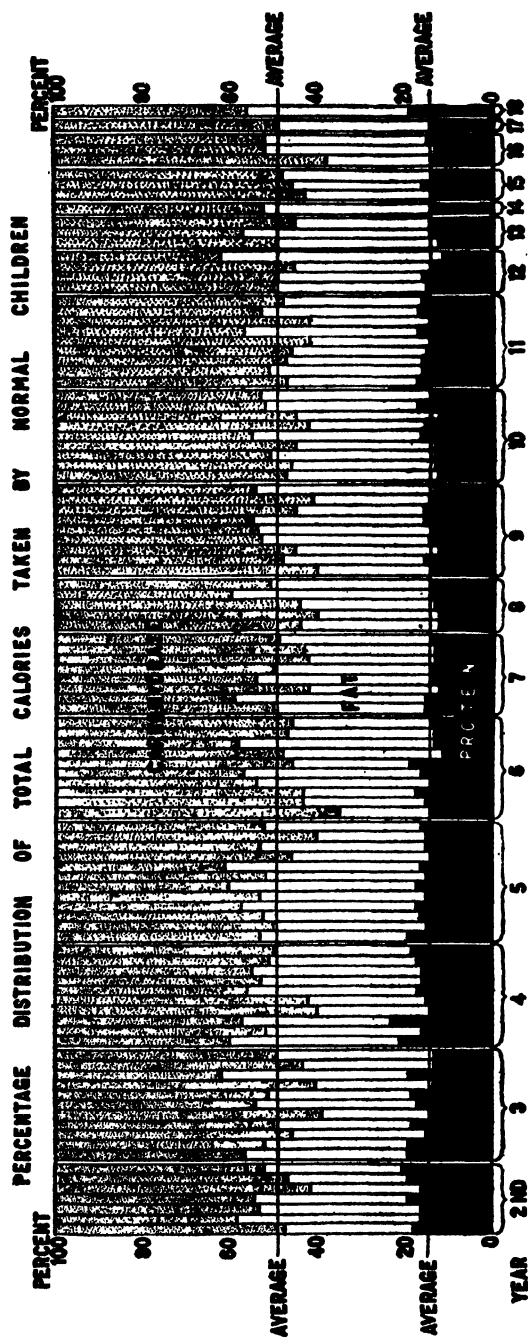


FIG. 1.

numbers as 35, 50 and 15 per cent., respectively. Therefore, healthy children were found to take, on the average, practically one-half their calories as carbohydrate, a little more than one-third as fat, and only one-sixth as protein. Since 1 gm. of fat has two and one-fourth times the caloric value of 1 gm. of protein, it is evident that in these proportions the amounts of fat and protein are practically equal, while the amount of carbohydrate is a little more than three times as great as either the fat or protein.

The total calories needed by a child of average activity at different ages have been estimated by us in a former article.¹ Using the average distribution of the calories given above, the theoretical requirements in fat, carbohydrate and protein have been calculated. Charts 2 and 3 show this distribution of the total daily calories for both sexes throughout the period of growth. Charts 4 and 5 give for both sexes the distribution of the calories per kilo.

Table II gives the average daily requirement in grams of fat, carbohydrate and protein calculated according to this distribution. Table III gives the grams per kilo. From these tables it is seen that the results arrived at in this way agree very closely with the estimated requirements of fat, protein and carbohydrate given in the detailed discussion of the theoretic needs for each of these constituents.²

The variation from the average in total calories which are needed by individual children is considerable. This will undoubtedly cause some variation in the distribution of the calories. If the total caloric requirement is increased because of more than average activity, this increase may be met, as has been previously stated, by raising the carbohydrate intake alone. This would increase the proportion of carbohydrate above the average and reduce the proportion of fat and protein, although the amounts of fat and protein remain unchanged. When the total caloric need is lessened because a child is overweight for height or oversize for age, all three constituents of the diet may be reduced proportionally, the normal distribution of calories being retained. This is because the diminished caloric need for growth permits a reduction in the fat and protein as well as in the total

¹ Holt, L. E., and Fales, H. L.: *Am. J. Dis. Child.* 21: 1 (Jan.) 1921.

² Holt, L. E., and Fales, H. L.: *Am. J. Dis. Child.* 22: 371 (Oct.) 1921.

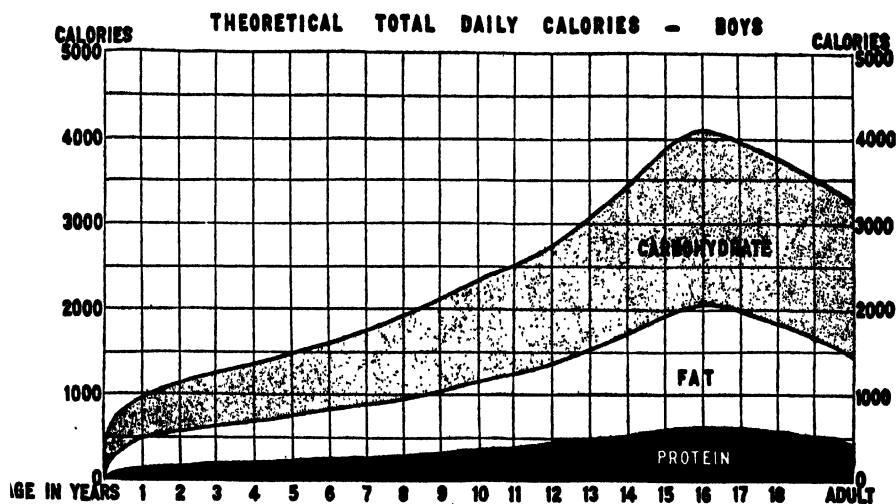


FIG. 2.

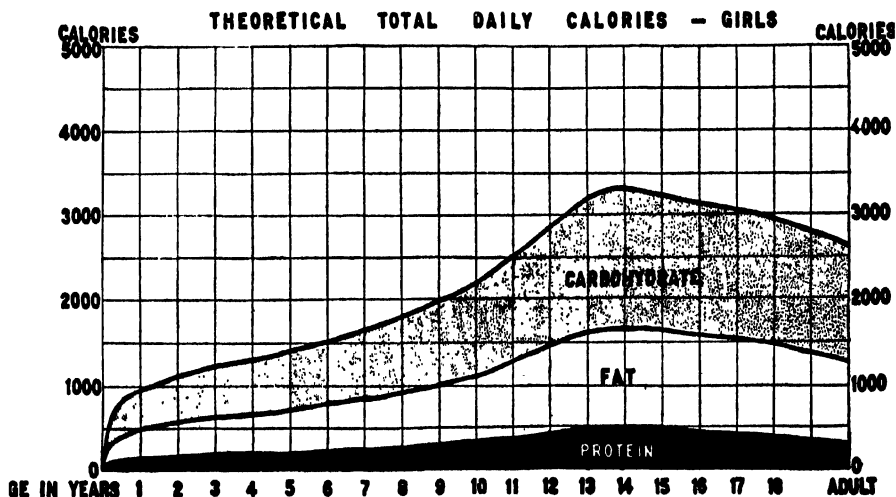


FIG. 3.

caloric requirement. On the other hand, when a child is undersize for age or underweight for height, the total caloric need is greater than the average, and in such cases the usual distribution of calories should be retained, all the constituents of the diet being increased.

In animal nutrition much emphasis has been placed on the importance of the "nutritive ratio" which is the relation of the sum of the fat and carbohydrate calories to the protein calories. The publi-

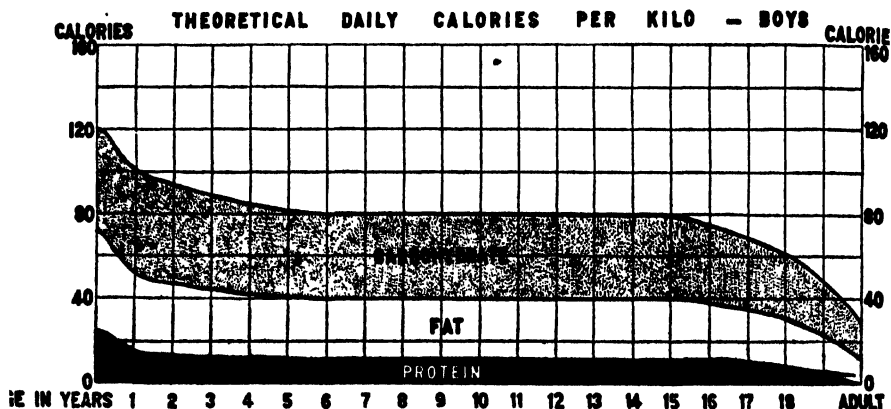


FIG. 4.

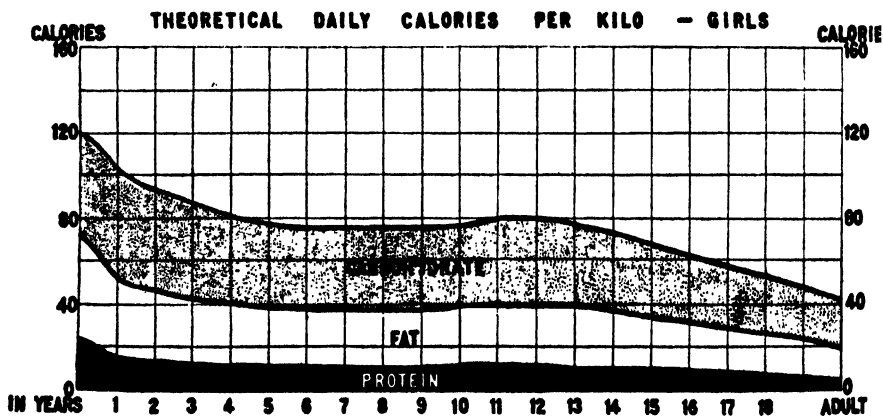


FIG. 5.

cations of the United States Department of Agriculture contain a great deal of information concerning the nutritive ratio which gives the best results in the feeding of animals. It is commonly held, according to Washburn and Jones,³ that a nutritive ratio of 8:1 or 9:1

³ Washburn, R. M., and Jones, C. H.: Vermont Agric. Exper. Sta. Bull. 195.

is proper for mature animals, while growing animals should receive a diet with a nutritive ration of 3:1 or 4:1.

The average diet of growing children, having a percentage distribution of calories of 35, 50 and 15, respectively, has a nutritive ration of 5.7:1. This value falls between that used for young animals and that used for mature animals. To obtain in the diet of

TABLE II.

Theoretical Average Requirements of Healthy Children—Grams Daily.

Age, years.	Boys.				Girls.			
	Calories, daily.	Fat, gm. daily.	Carbohydrate, gm. daily.	Protein, gm. daily.	Calories, daily.	Fat, gm. daily.	Carbohydrate, gm. daily.	Protein, gm. daily.
1	950	35.8	115.8	35.0	940	35.4	114.7	34.5
2	1,135	42.8	138.3	41.5	1,110	41.8	135.3	40.5
3	1,275	48.0	155.5	47.0	1,230	46.4	150.0	45.0
4	1,380	52.0	168.3	50.5	1,300	49.0	158.5	47.5
5	1,490	56.2	181.8	54.5	1,410	53.2	172.0	52.0
6	1,600	60.3	195.2	58.5	1,520	57.3	184.5	56.0
7	1,745	65.8	212.8	64.0	1,660	62.6	202.2	61.0
8	1,920	72.5	234.2	70.5	1,815	68.5	221.3	66.5
9	2,110	80.0	257.5	78.0	1,990	75.0	242.8	73.0
10	2,330	87.7	284.0	85.5	2,195	82.5	268.0	80.0
11	2,510	94.5	306.0	92.0	2,520	95.0	309.8	92.5
12	2,735	103.0	333.5	100.0	2,865	108.0	349.5	105.0
13	3,040	114.5	371.0	111.0	3,210	121.0	391.7	118.0
14	3,400	128.0	414.8	125.0	3,330	125.5	406.0	122.0
15	3,855	145.0	470.0	142.0	3,235	122.5	394.5	119.0
16	4,090	154.0	499.0	150.0	3,160	119.0	385.2	116.0
17	3,945	148.5	481.5	145.0	3,060	115.5	373.2	112.0
18	3,730	140.5	455.0	137.0	2,950	111.0	360.0	108.0
Adult	3,265	123.0	400.0	120.0	2,640	100.0	322.0	97.0

young children the nutritive ratio used in feeding young animals, that is, 3:1 or 4:1, it would be necessary for the child to take from 20 to 25 per cent. of the total calories as protein. So large an intake of protein is not, according to our observations, usual in the diet of healthy children, and it seems probable that a diet with a nutritive ratio of 5:1 or 6:1 provides sufficient protein for the growing child.

Animals grow much more rapidly than children. Furthermore, the normal activity of young animals in most cases equals or even

exceeds that of the mature animals. This activity gives rise to a demand for early muscular development. Both these factors, rapid growth and early muscularity, cause an increased need for protein in the diet.

The ration usually considered standard for adults consists of 100 gm. fat, 400 gm. carbohydrate and 100 gm. protein daily. This gives

TABLE III.

Theoretical Average Requirements of Healthy Children—Grams per Kilo Daily.

Age, years.	Boys.				Girls.			
	Calories, per kg.	Fat, gm. per kg.	Carbohy- drate, gm. per kg.	Protein, gm. per kg.	Calories, per kg.	Fat, gm. per kg.	Carbohy- drate, gm. per kg.	Protein, gm. per kg.
1	100	3.8	12.2	3.7	101	3.8	12.3	3.7
2	93	3.5	11.3	3.4	94	3.5	11.5	3.4
3	88	3.3	10.7	3.2	87	3.3	10.6	3.2
4	84	3.2	10.2	3.1	82	3.1	10.0	3.0
5	82	3.1	10.0	3.0	78	2.9	9.5	2.9
6	80	3.0	9.8	2.9	76	2.9	9.3	2.8
7	80	3.0	9.8	2.9	76	2.9	9.3	2.8
8	80	3.0	9.8	2.9	76	2.9	9.3	2.8
9	80	3.0	9.8	2.9	76	2.9	9.3	2.8
10	80	3.0	9.8	2.9	77	2.9	9.3	2.8
11	80	3.0	9.8	2.9	80	3.0	9.8	2.9
12	80	3.0	9.8	2.9	80	3.0	9.8	2.9
13	80	3.0	9.8	2.9	79	3.0	9.7	2.9
14	80	3.0	9.8	2.9	74	2.9	9.0	2.9
15	80	3.0	9.8	2.9	67	2.5	8.2	2.5
16	75	2.8	9.2	2.7	62	2.3	7.6	2.3
17	69	2.6	8.4	2.5	58	2.2	7.1	2.1
18	62	2.3	7.6	2.3	56	2.1	6.8	2.0
Adult	48	1.8	5.9	1.8	44	1.7	5.4	1.6

a distribution of the total calories as follows; fat, 31 per cent.; carbohydrate, 55 per cent.; protein, 14 per cent. The average diet of soldiers in the United States Army training camps, as computed by Murlin,⁴ had very nearly the same percentage distribution viz; fat, 31 per cent.; carbohydrate, 56 per cent.; protein, 13 per cent. This distribution does not differ very widely from the average we have found in the diets of children, that is, 35, 50 and 15, respectively.

⁴ Murlin, J. R., and Hildebrandt, F. M.: Am. J. Phys. 49: 531, 1919.

The evidence which has been presented, however, shows that children have greater needs for fat and protein than adults. Hence, it seems necessary to supply a higher proportion of fat and of protein than is allowed in the adult ration.

The reports published by other authors of actual diets taken by children show, as would be expected, considerable variation in the percentage distribution of the calories. In most cases, however, the protein taken ranges from 15 to 20 per cent. of the total calories. When the total calories were very high, the excess was nearly always in the form of carbohydrate, so that the proportion of protein was low, in some cases as low as 11 per cent. of the total calories. This occurred in the diets reported by Müller⁵ and in the average intake of the adolescent boys studies by Gephart.⁶

The proportion of calories supplied by fat in reported diets varies widely but in most cases lies between 30 and 40 per cent. An exception is found in the observations reported by Camerer,⁷ in which the fat supplied only from 20 to 30 per cent. of the total calories, while the proportion furnished by carbohydrate was very high, usually between 60 and 65 per cent.

In Camerer's schedule for the feeding of the child throughout the period of growth the proportions of the different constituents of the diet are varied with increasing age. He recommends giving 37 per cent. of the calories as fat at 1 year but reduces the allowance gradually to 23 per cent. at 5 years of age, maintaining this value throughout the remainder of growth. This reduction in fat is met by increasing the carbohydrate. In his schedule, the proportion of carbohydrate rises from 45 per cent. of the calories at 1 year to 60 per cent. at 5 years and remains at this figure throughout the remainder of the growth period. His protein allowance at 1 year supplies 18.5 per cent. of the total calories. The proportion is diminished to 17 per cent. at 5 years and remains at this figure during the remainder of growth. His distribution throughout the greater part of the growth period, therefore, may be expressed as 23, 60 and 17.

⁵ Müller, E.: *Biochem. Ztschr.* 5: 143, 1907.

⁶ Gephart, F. C.: *Boston M. & S. J.* 176: 17, 1917.

⁷ Camerer, W.: *Stoffwechsel des Kindes*, 1896.

The diet schedule for young children suggested by Steffen⁸ allows a very high proportion of the calories as fat, more than 50 per cent. up to 3 years and more than 40 per cent. through the seventh year.

Uffelmann⁹ suggests for young children diets which have a distribution of calories very similar to our average, the fat a little lower and the protein a little higher. Very few of the other observers who have made recommendations as to total caloric intake have suggested how this should be distributed as fat, carbohydrate and protein.

The conclusion to be drawn from all the observations and discussions presented in this series of articles is the emphasis to be placed on the importance of the balanced ration. We have shown that the protein need of the growing child is very important and there seems to be fairly general unanimity as to the amount required. All evidence seems to indicate that when 15 per cent. of the theoretical total caloric need is supplied by protein, the nutritive needs in protein are met. The amount of fat required is still a subject of debate but it seems reasonable and desirable that the amount of fat given to the growing child should be equal to the amount of protein. Since fat has two and one-quarter times the caloric value of protein, equal amounts of fat and protein would supply calories in the proportion of 2.25:1. Therefore, when the protein furnishes 15 per cent. of the total calories, an equal amount of fat would furnish about 35 per cent. The remaining caloric need, that is, 50 per cent. of the total calories, would then be supplied by carbohydrate.

When this distribution is deviated from to any marked degree for a long period, that is, when what seems to be a proper balance of the diet is disturbed, various undesirable results may follow. If the protein is reduced much below 15 per cent. of the caloric requirement, the nutritive need may not be met. If the fat given supplies much less than 35 per cent. of the total calories it usually happens that the carbohydrate intake is excessive and disturbance of digestion may follow. If the fat given supplies too high a proportion of the calories the fat tolerance of the child may be exceeded and digestive disturbance may result from the excess of fat. High fat with high protein

⁸ Steffen, W.: *Jahrb. f. Kinderh.* 46: 332, 1898.

⁹ Uffelmann, J.: *Hygiene des Kindes*, 1881.

forms a food combination likely to cause constipation. Low fat with high carbohydrate is apt to cause looseness of the bowels. A large carbohydrate intake means a reduction in the necessary protein and probably equally important fat.

On the whole, one may conclude both from theoretical considerations and from practical observation that a diet in which the fat supplies 35 per cent. of the total calories, the protein 15 per cent. and the carbohydrate 50 per cent., is one which meets the nutritive needs of the child after infancy, which is well borne by the digestive system and may be considered a well balanced ration.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

III. THE ALKALI-BINDING AND BUFFER VALUES OF OXYHEMOGLOBIN AND REDUCED HEMOGLOBIN.

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(Received for publication, July 22, 1922.)

The general problem of which the present paper attempts to solve a portion has been outlined in the first of these studies (1). The objects of the present work have been to determine quantitatively, on solutions of hemoglobin free from other proteins, the following data.

1. The amounts of base bound by oxyhemoglobin and reduced hemoglobin, respectively, at normal blood pH.

2. The changes in the base bound by oxyhemoglobin and reduced hemoglobin with changing pH (the buffer value, $\frac{dB}{dpH}$ in the sense outlined by Van Slyke (2)).

3. The nature of the curve relating oxygen increase to the increase in base bound at constant pH that occurs as reduced hemoglobin adds varying amounts of oxygen.

The unsatisfactory state of the present knowledge of the base-binding and buffer values of hemoglobin may be seen by referring to previous theoretical papers (2, 3) in which data for only the roughest approximate estimations were found available.

The increase in blood of the CO₂-replacing power (which is equivalent to increase in base-binding power) of hemoglobin when changed from the reduced to the oxygenated form was discovered by Christiansen, Douglas, and Haldane (4) and its physiological significance in the CO₂-carrying power of the blood was clearly pointed out. These authors published a single pair of curves, one of oxygenated,

the other of reduced blood, obtained by saturating samples of blood drawn at different times from the same individual. The blood was analyzed by methods which, as pointed out in our first paper (1), like those used by ourselves until the requirements of the present work forced the development of refinements, were adequate only to give approximate knowledge of the magnitude of the changes involving as a maximum 6 or 7 volumes per cent of CO_2 . Up to the present the single pair of curves, published by Christiansen, Douglas, and Haldane and a similar pair published by Joffe and Poulton (5) have remained almost the entire experimental basis of knowledge concerning the mutual effects of oxygen and carbon dioxide tensions in the blood.

Concerning the nature of the intermediate relationships (*i.e.* whether the first portion of oxygen taken up by reduced hemoglobin has more or less effect on its base-binding power than the last portion), Christiansen, Douglas, and Haldane have tacitly assumed in their calculations explaining the physiological significance of the phenomenon, that the change is a linear one, the first portion of oxygen taken up having the same relative effect in CO_2 replacement as the last. The same assumption has been made by L. J. Henderson (6), Van Slyke (3), and Peters, Barr, and Rule (7), although admittedly without experimental basis.

The assumption is in fact an essential part of Henderson's theory explaining the O_2 - CO_2 effect on hemoglobin by assuming that the oxygenated form is an acid, HHbO_2 , with a greater dissociation constant than the reduced form, HHb . If this is the case the proportion of alkali bound by each form is, at a given pH, fixed in accordance with the equation $\text{H}^+ = K'_o \frac{[\text{HHbO}_2]}{[\text{BHbO}_2]}$ and $\text{H}^+ = K'_r$

$\frac{[\text{HHb}]}{[\text{BHb}]}$ K_o and K_r representing the dissociation constants of the

carboxyl groups of oxygenated and reduced hemoglobin affected by the change in oxygenation. Under these conditions, at a given pH, the alkali bound by hemoglobin would change in exact proportion to

the oxygen taken up, the ratio $\frac{dB}{d[\text{HbO}_2]}$ at a given pH being constant.

The data in the present paper confirm the above assumptions as to the straight line form of the curve relating changes in oxygen to changes in base bound by hemoglobin.

We have found that satisfactory settlement of the questions outlined in the introduction, but especially of the third, demanded technique of a different order of accuracy from that available when the problem was at first attempted. Finally with the development of Heidelberg's method (8) for preparing readily and in large amounts crystalline hemoglobin practically electrolyte-free, with the attainment of an accuracy of about 1 part in 500 in blood gas analyses by means of the "constant volume" apparatus (9), and with the evolution of the general technique for saturation and handling of blood and hemoglobin solutions outlined in the first paper, especially the use of the double-chambered tonometer described there under the "first saturation method," our technique became refined in the necessary points to such a degree that reproducible quantitative results could be obtained. It will be seen that the added errors of manipulation and of the three analyses involved, *viz.*, blood O_2 , blood CO_2 and CO_2 tension in the final gas mixtures, cause deviations on the average of not over 0.1 mm. of $BHCO_3$ (0.2 volume per cent of CO_2), from the mean linear curve, although each experiment shows one or two points with somewhat wider deviation.

EXPERIMENTAL.

The hemoglobin crystals were prepared by Heidelberg's (8) method, and used as soon as possible after they had been twice recrystallized. They were dissolved in water to which NaOH was added to make the Na concentration indicated in the tables, 0.04 N in Experiment 1, 0.03 N in Experiment 2, 3, and 4. It will be seen from the table headings that the preparations all fell somewhat short of 100 per cent of their theoretical oxygen capacity. This is not due to admixture of other substances, but to the extreme readiness of hemoglobin to tautomerize in some manner and lose some of its ability to bind labile oxygen. If dried, even at high vacuum and in the cold, hemoglobin is almost completely inactivated, although in solubility and color it still resembles oxyhemoglobin. Slow loss of activity occurs even while standing in solution. The maximum loss

in our preparations was one-fifth of the theoretical oxygen capacity. There is no reason to expect such inactivation of part of the hemoglobin to affect the alteration of the rest in base-binding power when oxygen is added or lost. The presence of inactive hemoglobin in our preparations, however, increases in a way discussed later, the limit of error that must be allowed in estimating the absolute amounts of base bound per mol of oxyhemoglobin and reduced hemoglobin.

The hemoglobin was dissolved to make about the same concentration found in horse blood, about 7 mM. (equivalent to 15 to 16 volumes per cent of oxygen capacity), and the amount of alkali added was such that the system at physiologically normal CO_2 tensions had pH values within physiological limits.

The saturations were performed at 38° with the double tonometer elsewhere described (1). Usually 20 or 30 cc. of solution were saturated with 300 cc. of gas. The lower chamber of the tonometer was filled with solution and clamped off from the upper. When low oxygen tensions were desired the upper chamber was evacuated with a water pump and washed out three times with hydrogen before the final gas mixture was let in. After 20 minutes saturation the gas mixture was renewed, in order to make certain that the final conditions should approximate as closely as possibly those desired. After preliminary experiments the behavior of the hemoglobin was known well enough to permit rather close prediction of the pH, $[\text{HCO}_3^-]$, and $[\text{HbO}_2]$ values that would be obtained.

One of our main purposes was to ascertain the change in alkali bound when the oxygen saturation of the hemoglobin was varied, the pH being kept constant. It was impracticable so closely to control conditions that a series of solutions with varying oxygen and CO_2 tensions should all be brought to exactly the same pH, although it could be approximated. Having determined the base bound by the hemoglobin at the pH obtained, say 7.27 instead of a desired 7.30, the base bound at 7.30 was estimated by extrapolation. This was performed by determining the slope of the B, pH line (B representing the total base bound by hemoglobin), by means of several points on completely oxygenated solutions with varying CO_2 tensions. The slope, as shown in the following paper, increases with increasing oxygenation, but the change is small enough to permit neglecting it

for short distance extrapolations. Consequently, lines were drawn through the reduced and intermediate points parallel to the line on which the oxygenated points lay, and the intersections of these lines with the desired pH line were used to estimate the base bound at that pH by hemoglobin oxygenated to the extent ascertained. Examples of this mode of extrapolation are seen in Figs. 2a, 3a, and 4a.

From the base bound by hemoglobin thus ascertained at a given pH with varying degrees of oxygen saturation a curve was drawn showing the relation of oxygen content to the base bound at the given pH (Figs. 2b, 3b, 4b).

The CO_2 tensions were determined as described in connection with the saturation method (1) by analysis by Haldane's method of the separated gases in the upper chamber.

The CO_2 contents of the solutions were determined by means of the "constant volume" apparatus (9). The apparatus used had a total volume of 100 cc. The gas was measured at 5 cc. volume, which could be read with an error not exceeding 1 part per 1,000. Samples of either 2 or 3 cc. of solutions were taken and the changes in pressure read were in the neighborhood of 100 to 160 mm. with 2 cc., and 150 to 240 mm. with 3 cc. samples. The accuracy with which duplicates could be obtained may be indicated by the following series, obtained in the course of Experiment 3 by Neill. Expressed in millimols of total CO_2 the results were:

Saturation 1, 15.40 and 15.31; Saturation 2, 20.17 and 20.12; Saturation 3, 22.54 and 22.47; Saturation 4, 22.07 and 22.02; Saturation 5, 19.12 and 19.10; Saturation 6, 19.10 and 19.08; Saturation 7, 18.80 and 18.70; Saturation 8, 18.93, 18.73, and 18.76; Saturation 9, 21.50 and 21.45; and Saturation 10, 21.07 and 21.11. In the above series 2 cc. samples were used. When duplicates failed to agree within 0.1 mm. as in Saturation 8, a third sample was analyzed. Excluding the one obviously faulty first analysis of No. 8, the average difference between duplicates was 0.05 mm. Later when 3 cc. samples were used, the average error fell to 0.03 mm. which is about 1 part in 500 of the values determined.

For the oxygen determination in the solutions an apparatus with a total volume of 50 cc. was used. The pressures were measured with the gas volume at 2.0 cc. for oxygenated blood, and at 0.5 cc. for

blood near complete reduction. The oxygen was determined from the change in pressure before and after absorption with pyrogallol. The maximum deviation of duplicates accepted was 0.1 mm., but analyses were usually repeated if 0.05 mm. was exceeded.

The maximum difference accepted in the duplicate Haldane analyses of the gas phase was 0.04 per cent.

If the difference between duplicates exceeded the values indicated as acceptable, and other analyses could not be done, the results of both duplicates are given in the table.

In calculating pH, if an error of 1 part per 100 occurred in the Haldane determination of the CO_2 tension in the gas and of 1 part per 200 in the CO_2 determination in the solution, and both errors were in the opposite direction, their combined effect on the pH would be a change of 0.007, which may be taken as the maximum error assignable to the estimations of pH changes. Our curves indicate that the actual error was usually less.

The sets of analyses in each table are numbered in the order in which the solutions were saturated.

Symbols Used.

The following, for the most part already familiar, symbols are used. $[\text{BHCO}_3]$, $[\text{H}_2\text{CO}_3]$, $[\text{CO}_2]$ for concentration of NaHCO_3 , H_2CO_3 and total CO_2 , respectively, in the solutions; $[\text{O}_2]$ for the total oxygen concentration; $[\text{HbO}_2]$ for the concentration of oxygen combined with hemoglobin; $[\text{Hb}]$ for the concentration of reduced hemoglobin; C for the concentration of total hemoglobin in all forms; $[\text{BHbO}_2]$ and $[\text{BHb}]$ for concentrations of univalent base bound by oxyhemoglobin and reduced hemoglobin, respectively; B for the total base bound in both forms. All these concentrations are expressed in millimolar (mm.) terms, the molecular concentrations of C , HbO_2 , and Hb being taken equal to that of the maximum oxygen with which the hemoglobin is combined or could combine.

The carbon dioxide and oxygen tensions in mm. of mercury are expressed as p_{O_2} and p_{CO_2} .

The molecular buffer values of oxyhemoglobin and reduced hemoglobin are indicated as β_o and β_r respectively.

The value calculated for the molecular buffer effect is independent of the concentration unit used, as long as the same unit is used for both B and C in the ratio $\frac{dB}{Cd pH}$. In this paper we have for convenience expressed concentrations in millimolecular instead of molecular units, but the β_0 values are the same as when M is the unit of concentration. This is not, of course, true of the $\frac{dB}{dpH}$ ratio, which when expressed in millimolar terms, as in this paper, is 1,000 times as great as when expressed as molar units. A $\frac{dB}{dpH}$ blood value of 20 in this paper therefore corresponds to one of 0.020 in Van Slyke's paper on buffers (2).

Calculations.

The calculation of $[BHCO_3]$ from the total $[CO_2]$ and the p_{CO_2} was according to Formula 2, of Table IV, of our first paper (1). The calculation of pH from CO_2 and p_{CO_2} was made according to Formula 3 of the same table. The total base bound by hemoglobin, $[BHbO_2] + [BHb]$, was obtained by subtracting the $[BHCO_3]$ from the total base added. For example, in Table III, the total base added was 30.00 mM. The $[BHCO_3]$ in Solution 1 was 14.49. Hence the remainder of base, bound by the hemoglobin (since no other acids were present), was $30.00 - 14.49 = 15.51$.

Constants.

The value of pK' in the equation $pH = pK' + \log \frac{[BHCO_3]}{[H_2CO_3]}$ was determined experimentally on several hemoglobin solutions by determining in reduced solutions the $[H_2CO_3]$ and $[BHCO_3]$ as above described, and the pH electrometrically. The results given in Table V, indicate, as the average value, $pK' = 6.18$.

The solubility coefficient for CO_2 , $\alpha_{CO_2} = 0.531$ (given in Table V, first paper (1)), was estimated as follows: Blood, plasma, and hemoglobin were acidified to pH 4.3 with HCl and the CO_2 content was determined after saturating with pure CO_2 at atmospheric tension. The determinations were made on 5 cc. samples, which were

transferred to the constant volume apparatus without exposure to air. The error was about 1 part in 500, and we obtained theoretical results for α_{CO_2} in pure water. For blood and plasma we found at 38° α_{CO_2} values 0.501 and 0.531, respectively. Each was 0.10 below Bohr's (10) estimate made on the assumption that hydrogen and

TABLE I.

Crystalline Horse Hemoglobin No. 5.

Total hemoglobin content of solution 7.97 mm. Colorimetric.

Oxygen capacity of solution 6.89 "

Oxygen capacity _____ 0.854

Total hemoglobin content

Conductivity of saturated HbO_2 solution in H_2O . 4.3×10^{-3} mhos.

Concentration of NaHCO_3 added to solution 40 mm.

Equilibration Data.

No.	P_{CO_2}	Total $[\text{CO}_2]$	$[\text{BHCO}_2]$	$[\text{BHbO}_2]$	pH
	mm.	mm.	mm.	mm.	
1	23.3	20.75	20.02	19.98	7.541
2	26.3	21.40	20.58	19.42	7.50
3	31.0	22.58	21.61	18.39	7.45
4	42.4	24.87	23.55	16.45	7.35
5	41.6	24.80	23.50	16.50	7.358
6	46.4	25.66	24.21	15.79	7.325
7	54.1	26.95	25.27	14.73	7.276
8	55.9	27.31	25.57	14.43	7.268
9	61.1	28.00	26.10	13.90	7.237
10	101.0	32.17	29.03	10.97	7.066

CO_2 have the same relative solubilities in blood as in water. We have reason to believe that Bohr's estimate was close, and that our method, perhaps because of hydration of the acidified proteins, gives slightly too low results. With 7 mm. hemoglobin plus NaOH to 0.03 N concentration we found by our method an α_{CO_2} of 0.521. Adding the 0.10 correction, apparently inherent in our method, gives 0.531.

EXPLANATION OF FIGURES.

In Figs. 1, 2*a*, 3*a*, and 4*a* the relations between pH and base B bound by oxyhemoglobin are shown in each case by the slope of the

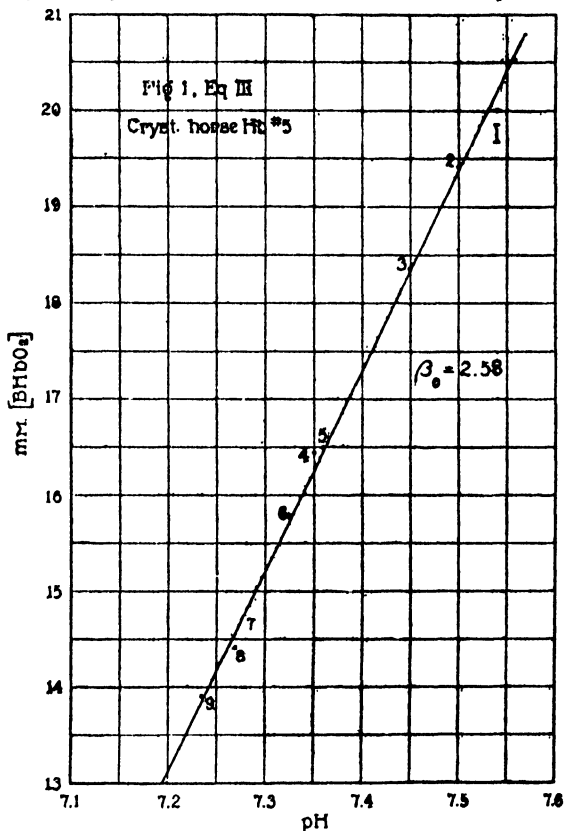


FIG. 1. From the line through the oxygenated points 1 to 9, $\frac{dB}{dpH}$ is calculated as 23.0, and β_0 as 2.58.

line through the three or more points representing oxygenated solutions. The molecular buffer effect β_0 is calculated as $\frac{dB}{Cd \text{ pH}}$, where $\frac{dB}{dpH}$ is the slope of the line through the oxygenated points, and C is the concentration of hemoglobin in the solution.

In Figs. 2a, 3a, and 4a the effect of varying degrees of oxygenation on the base bound by hemoglobin at constant pH is shown by the levels at which lines through points representing oxygenated, reduced, and partially reduced solutions cut a given pH line. The values

TABLE II.

Crystalline Horse Hemoglobin No. 7.

Total hemoglobin content of solution	6.71 mm.	Colorimetric.
	7.13 "	Kjeldahl.
	6.92 "	Mean.
Oxygen capacity of solution	5.50 "	
Oxygen capacity	0.795	
Total hemoglobin content		
Conductivity of saturated HbO ₂ solution in H ₂ O.	3.8×10^{-5}	mhos.
Concentration of NaOH added to solution	30 mm.	

Equilibration Data.

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	Total [CO ₂]	[BHCO ₂]	[BHb] + [BHbO ₂]	pH	[BHb] + [BHbO ₂] at pH 7.30.
	mm.	mm.	mm.	mm.	mm.	mm.	mm.		mm.
1	143.1	5.52	5.33	29.5	16.20	15.28	14.72	7.403	12.80
2	137.5	5.25	5.07	57.3	20.72	18.94	11.06	7.207	
3	131.8	5.18	5.01	84.3	24.01	21.39	8.61	7.092	
5*	10.0	1.74	1.73	43.7	20.48	19.12	10.88	7.328	10.28
6	20.0	3.08	3.05	44.1	20.13	18.75	11.25	7.315	10.95
7	34.5	4.12	4.08	45.0	19.55	18.15	11.85	7.292	11.93
					19.66	18.26	11.74	7.295	
8	28.4	3.86	3.83	57.4	21.55	19.76	10.34	7.224	11.75
9	5.7	1.05	1.04	43.2	20.78	19.43	10.57	7.340	9.70

* No. 4 was lost.

indicated by these intersections are given in the last columns of Tables II, III, and IV.

In Figs. 2b, 3b, and 4b, the amounts of base indicated by the above described intersections are plotted against amounts of oxygen combined with the hemoglobin. The curves in all three cases are straight lines. The values of the molecular increments of base added by the

hemoglobin at the given pH per molecular increment of oxygen are indicated as $\frac{dB}{d[HbO_2]}$.

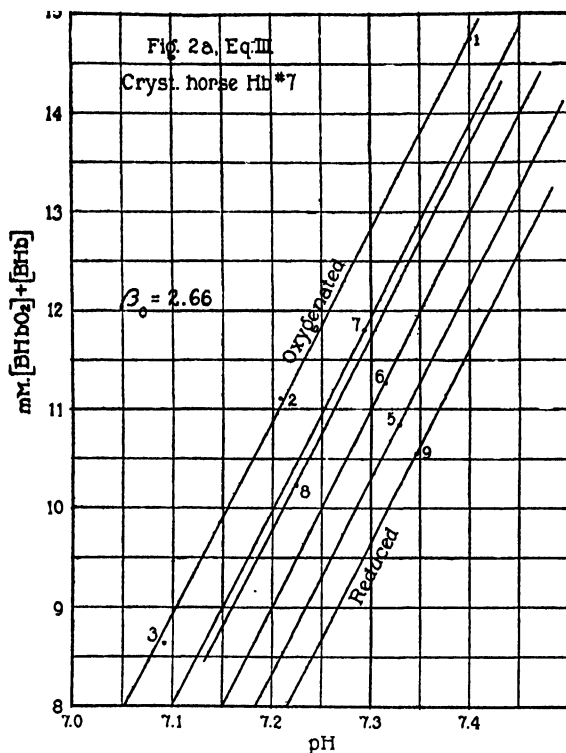


FIG. 2a. From the line through the oxygenated points 1, 2, and 3, $\frac{dB}{dpH}$ is calculated as 18.4, and β_0 as 2.66.

Intersections of lines through the other points with the pH 7.30 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table II from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.30 line are given.

DISCUSSION OF RESULTS.

It is convenient to discuss here in their reverse order the three questions put at the beginning of this paper.

The Nature of the Curve Relating Increment of Oxygen Bound to Increment of Base Bound by Hemoglobin at Constant pH.

As shown by Figs. 2b, 3b, and 4b, the $\frac{dB}{d[\text{HbO}_2]}$ curve is a straight line.

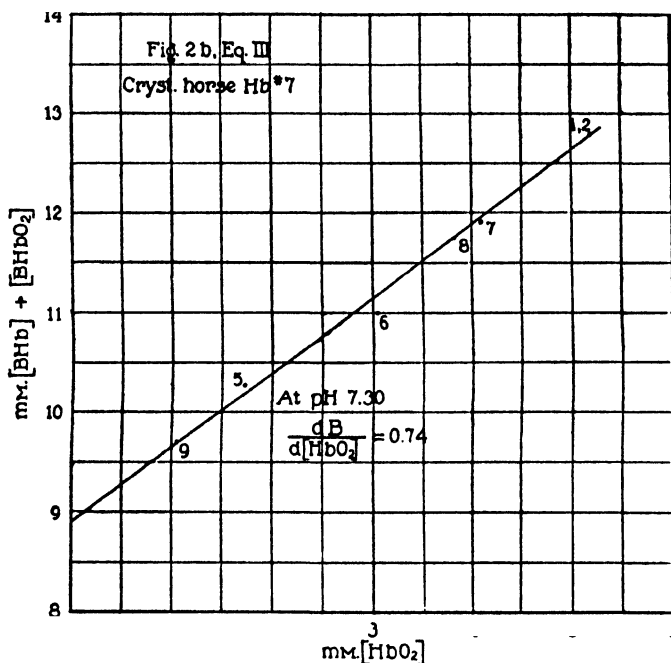


FIG. 2b. Amounts of base bound by hemoglobin at pH 7.30 compared with amounts of oxygen bound. Data from Table II and Fig. 2a.

From the slope of the line, $\frac{dB}{d[\text{HbO}_2]}$ is calculated as 0.74.

The Buffer Value of Oxyhemoglobin over the Physiological pH Range, and the Indicated Minimum Number of Monovalent Acid Groups Active as Buffers at That Range.

a. Oxyhemoglobin.—By Figs. 1, 2a, 3a, and 4a the B, pH lines for oxygenated hemoglobin solutions are shown to be straight over at least the range pH 7.2 to 7.5. The values of the molecular buffer ratios were $\beta_0 = 2.58, 2.66, 2.68$, and 2.63 , average 2.64 .

It has been previously shown (2) that the maximum molecular buffer value, β_m , of one monovalent acid group is 0.575. The minimum number of acid groups in a polyvalent acid that are acting as buffers at a given pH is therefore indicated by dividing the observed

TABLE III.

Crystalline Horse Hemoglobin No. 11.

Total hemoglobin content of solution	7.73	mm. Colorimetric.
	7.54	" Kjeldahl.
	7.64	" Mean.
Oxygen capacity of solution	7.13	"
Oxygen capacity	0.933	
Total hemoglobin content		
Conductivity of saturated HbO ₂ solution in H ₂ O	3.8×10^{-5}	mhos.
Concentration of NaOH added to solution	30	mm.

Equilibration Data.

No.	P_{O_2}	Total [O ₂]	[HbO ₂]	P_{CO_2}	Total [CO ₂]	[BHCO ₂]	[BHb] + [BHbO ₂]	pH	[BHb] + [BHbO ₂] at pH 7.31.
	mm.	mm.	mm.	mm.	mm.	mm.	mm.		mm.
1	144.2	7.13	6.94	28.0	15.36	14.49	15.51	7.401	13.63
2	138.7	7.18	7.00	55.0	20.15	18.44	11.56	7.212	
				55.8		18.41	11.59	7.205	
3	135.0	6.96	6.78	72.9	22.51	20.24	9.76	7.130	
				73.4		20.23	9.77	7.127	
4	3.2	0.60	0.60	48.9	22.05	20.53	9.47	7.310	9.47
5	14.8	3.10	3.08	38.6	19.11	17.19	12.09	7.354	11.15
6		4.51	4.48						
	22.6	4.74	4.71	42.9	19.09	17.75	12.25	7.304	12.35
7	20.1	3.99	3.96	39.6	18.75	17.52	12.48	7.333	12.00
8	23.9	4.77	4.74	41.5	18.75	17.46	12.54	7.311	12.50
9	4.2	0.84	0.83	45.9	21.48	20.05	9.95	7.327	9.57
10	8.8	1.54	1.53	45.9	21.09	19.66	10.34	7.319	10.12

β_m value by 0.575. If we do this with oxyhemoglobin we obtain $\frac{2.64}{0.575} = 4.6$. It is evident that at least five buffer groups in the hemoglobin molecule are active over this range.

By "active" is meant that they are partially, but only partially, combined with alkali. The maximum buffer effect is exerted when a buffer acid is combined with half an equivalent of base (2). If there were five groups, each exerting maximum buffer value at a given pH, they would bind altogether 2.5 molecules of univalent

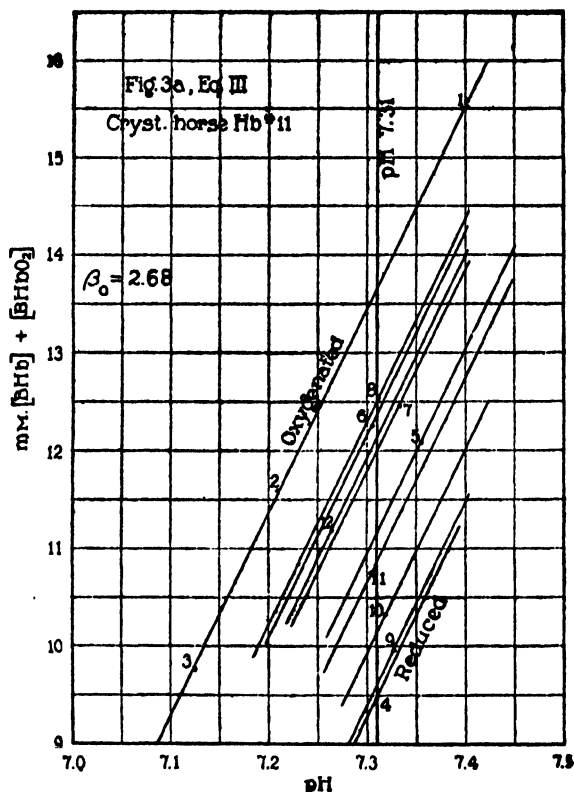


FIG. 3a. From the line through the oxygenated points 1, 2, and 3, $\frac{dB}{dpH}$ is calculated as 20.2, and β_0 as 2.68.

Intersections of lines through the other points with the pH 7.31 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table III from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.31 line are given.

alkali. As a matter of fact it will be shown that at pH 7.4 oxyhemoglobin does bind 2.1 equivalents of alkali. So it seems fairly probable that six or seven acid groups may be acting. That there are less than five is excluded. It is obvious that with the alkali distributed among five or more acid groups in the hemoglobin molecule it is impossible to calculate a single dissociation constant in the manner applicable to a monovalent acid.

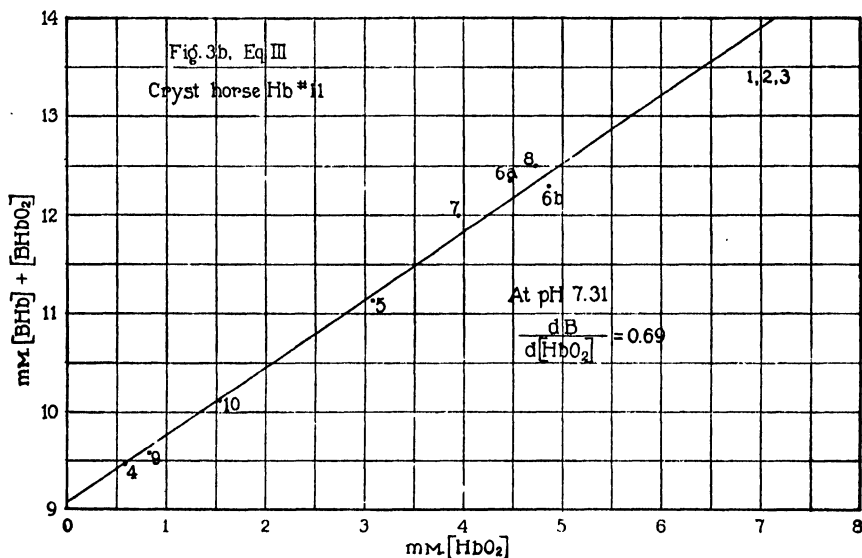


FIG. 3b. Amount of base bound by hemoglobin at pH 7.31 compared with amounts of oxygen bound. Data from Table III and Fig. 3a.

From the slope of the line, $\frac{dB}{d[HbO_2]}$ is calculated as 0.69.

b. Buffer Value of Reduced Hemoglobin.—Because of an interruption which will prevent resumption of our experiments for several months, we are publishing our other data before the direct determination of the value of β_r , from the slope of the $\frac{dB}{dpH}$ line in reduced solution of crystallized hemoglobin, has been completed. In Table VIII of the accompanying paper (11), however, we have estimated,

from accurately determined differences between the buffer value of blood in the oxygenated and reduced conditions, respectively, that if β_0 is 2.64 as determined in this paper, β_2 is 2.45.

TABLE IV.

Crystalline Horse Hemoglobin No. 14.

Total hemoglobin content of solution	7.25	mm. Colorimetric.
	7.54	" Kjeldahl.
	7.40	" Mean.
Oxygen capacity of solution	6.37	"
Oxygen capacity	0.860	
Total hemoglobin content		
Conductivity of saturated HbO ₂ solution in H ₂ O	3.9×10^{-5}	mhos.
Concentration of NaOH added to solution	30	mm.

Equilibration Data.

No.	P_{O_2}	Total [O ₂]	[HbO ₂]	P_{CO_2}	Total [CO ₂]	[BHCO ₂]	[BHb] + [BHbO ₂]	pH	[BHb] + [BHbO ₂] at pH 7.42.
	mm.	mm.	mm.	mm.	mm.	mm.	mm.		mm.
1	137.6			30.3	17.66	16.72	13.28	7.428	13.18
2	134.6	6.26	6.08	31.9	17.95	16.96	13.04	7.413	
3	143.9	6.22	6.03	60.7	22.58	20.69	9.31	7.220	
4	34.4	5.68	5.63	33.5	18.44	17.40	12.60	7.403	12.90
5	10.6	2.77	2.76	36.9	20.47	19.32	10.68	7.406	10.92
6	2.8	0.60	0.60	37.0	21.67	20.52	9.48	7.431	9.21
					21.78	20.63	9.37	7.433	
7	22.1	4.87	4.85	33.2	18.70	17.67	12.33	7.433	11.95
					18.87	17.84	12.16	7.437	

The Base Bound by Oxyhemoglobin and Reduced Hemoglobin at pH 7.4.

Oxyhemoglobin.—If we take the intersections with the pH 7.4 line, of the lines indicating base bound as BHbO₂ and passing through the completely oxygenated points on Figs. 1, 2*a*, 3*a*, and 4*a*, we find that the amounts of alkali bound have the values indicated in Table VI, the average being 2.09 atoms of Na bound by 1 molecule of oxyhemoglobin.

To the data taken from previous tables we have added those from two experiments with Preparations 6 and 10, which have not been reported in detail, because determinations of points of partial oxygenation were lacking. The percentages of hemoglobin capable of binding oxygen in these preparations were 97 and 92, respectively.

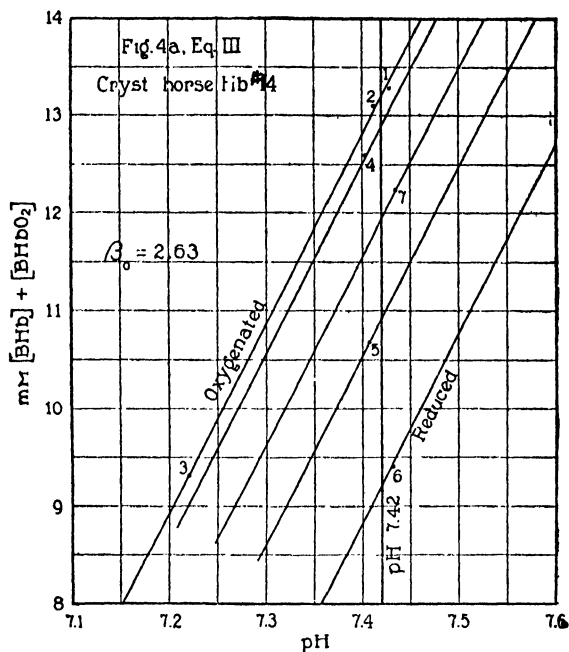


FIG. 4a. From the line through the oxygenated points 1, 2, and 3, $\frac{dB}{dpH}$ is calculated as 19.3, and β_0 as 2.63.

Intersections of lines through the other points with the pH 7.42 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table IV from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH 7.42 line are given.

It is probable that in Experiment 4 less alkali was added than was estimated. This error would not affect the determination of pH or β_0 or of the $\frac{dB}{d[HbO_2]}$ line, since these are all based on differences in

$[\text{BHCO}_3]$ and not on absolute values. It would invalidate calculations of the $[\text{BHbO}_2]$ value, however, and we have left this determination out in averaging the results.

Reduced Hemoglobin.—In Figs. 2*b*, 3*b*, and 4*b* the height of the B, $[\text{HbO}_2]$ lines at the abscissa representing complete reduction ($[\text{HbO}_2] = 0$) indicates the amounts of base bound by the completely reduced

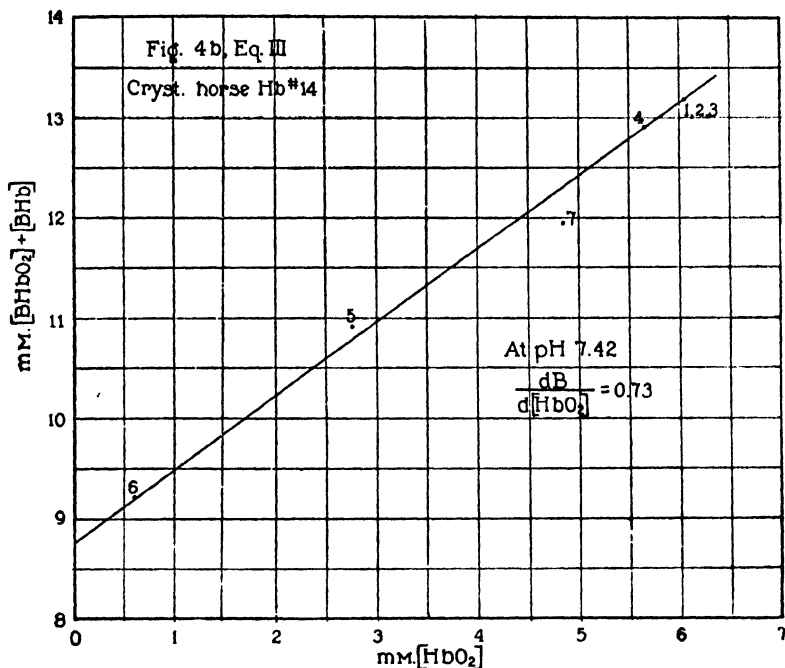


FIG. 4*b*. Amounts of base bound by hemoglobin at pH 7.42 compared with amounts of oxygen bound. Data from Table IV and Fig. 4*a*.

From the slope of the line, $\frac{dB}{d[\text{HbO}_2]}$ is calculated as 0.73.

hemoglobin. From the values thus found, in Figs. 2*b*, 3*b*, 4*b*, and similar figures representing data obtained from Preparations 6 and 10, the results in Table VII as "Na bound per mol Hb" are calculated. From these figures the data in the last column of Table VII, at pH 7.4, for comparison with the results in Table VI, are calculated.

TABLE V.

*Summary of Experiments to Determine pK' of Hemoglobin Solutions.**Technique:*

Double tonometer "First equilibration method." Double saturation.

Saturation at 38° with $H_2 + CO_2$.

Gas phase analyzed in Haldane apparatus.

Liquid phase analyzed for CO_2 .pH of liquid phase determined electrometrically at 38° at CO_2 tension of equilibration.*Solution:*Twice recrystallized HbO_2 plus NaOH to 30 mM.

Date.	Hb preparation.	Total B concentration.	Total Hb concentration.	P_{CO_2}	Total $[CO_2]$	$[HCO_2]$	$R = \frac{[HCO_2]}{[H_2CO_3]}$	log R	Electrometric pH.*	pK'
1922		mM.	mM.	mM.	mM.	mM.				
May 5	Horse 7	30	6.71	52.2	21.44	19.81	12.19	1.086	7.26	6.17
" 5	" 7	30	6.71	84.4	25.55	22.92	8.74	0.941	7.08	6.14
June 1	" 11	30	7.13	48.9	22.05	20.53	13.50	1.130	7.31	6.18
" 16	Dog 16	30	6.27	38.3	25.23	24.05	20.36	1.309	7.50	6.19
" 16	" 16	30	6.27	38.5	25.52	24.33	20.48	1.311	7.50	6.19
Average.....										6.18

* Electrometric pH determinations are based on 0.1 N HCl assuming 1.090 as its pH at 38°.

TABLE VI.

Base Bound by Oxyhemoglobin at pH 7.4.

Experiment No.	Preparation No.	Total hemoglobin content, C.	Na bound* as Na HbO_2 at pH 7.4.	Na bound per mol hemoglobin at pH 7.4 = $\frac{[Na HbO_2]}{C}$	α calculated = $7.4 - \frac{[Na HbO_2]}{2.64 C}$
		mM.	mM.	mol	
1	5	7.97	17.23	2.16	6.58
2	7	6.92	14.80	2.14	6.59
3	11	7.64	15.53	2.06	6.62
4	14	7.40	12.80	1.74	6.74
—	6	7.58	15.60	2.06	6.62
—	10	8.87	18.20	2.05	6.62
Average†.....				2.09	6.61

* By interpolation on line connecting oxygenated points of Figs. 1, 2a, 3a, and 4a.

† Preparation No. 14 left out of average.

The Difference Between the Base Bound by Reduced and by Oxygenated Hemoglobin.

The difference between the average equivalents of Na bound per molecule of oxygenated and reduced hemoglobin respectively is, from Tables VI and VII, $2.09 - 1.51 = 0.58$. However, the hemoglobin preparations used were on the average capable of taking up and giving off only 0.85 molecule of O_2 . So that the real average change of Na per mol change in combined O_2 was approximately $\frac{0.58}{0.85} = 0.68$ equivalents of Na.

TABLE VII.
Base Bound by Reduced Hemoglobin.

Experiment No.	Preparation No.	Total hemoglobin content, C.	pH	Na bound as [Na Hb] at pH indicated.	Na bound per mol Hb = $\frac{[\text{Na Hb}]}{C}$	b calculated as = $\frac{\text{pH} - [\text{Na Hb}]}{2.45^\circ \text{C}}$	Na estimated* bound at pH 7.40 per mol Hb.
		mm.		mm.	mol		mol
2	7	6.92	7.30	8.86	1.28	6.78	1.52
3	11	7.13	7.31	9.05	1.27	6.78	1.49
4	14	7.40	7.42	8.75	1.18	6.94	1.13
—	6	7.58	7.35	10.45	1.38	6.82	1.50
—	10	8.87	7.30	11.60	1.31	6.77	1.55
Average†.....						6.79	1.51

* Estimated by adding 2.45 ($7.4 - \text{pH}$) to Na bound at observed pH.

† Preparation No. 14 left out of average.

The values of the ratio $\frac{dB}{d[\text{HbO}_2]}$ determined in Experiments 2, 3, and 4, were 0.74, 0.69, and 0.73, respectively. It appears that we may state that the ratio is approximately 0.7 for horse hemoglobin. How much error in it we would allow for is not yet certain. It may be as great as ± 0.1 , but is probably not over ± 0.05 . We hope to establish it more accurately later.

The presence of inactive hemoglobin in our preparations widens the margin of error that must be allowed for in the determination of the absolute amounts of base bound per molecule of oxygenated and reduced hemoglobin. If the inactive hemoglobin has the base-binding power of oxyhemoglobin, then the average figure 2.09 given in

Table VI is correct, but this figure for reduced hemoglobin should be lowered by about 0.1. If, however, the inactive hemoglobin has the base-binding power of reduced hemoglobin, the completely oxygenated preparation must bind about 2.2 equivalents of base. The preparation of entirely inactive hemoglobin and the determinations of its base-binding powers are required to settle the nature of the correction it necessitates. Until this has been accomplished we must make allowance for the margin of error that may be occasioned in the figures for either oxygenated or reduced hemoglobin. Such allowance extends the range of results for reduced hemoglobin from 1.55–1.49 to 1.55–1.39 (or 1.47 ± 0.08 equivalents of base per molecule of hemoglobin). It extends the range of results for oxyhemoglobin from 2.05–2.16 to 2.05–2.26, or 2.15 ± 0.10 equivalents.

The Base Bound by Oxyhemoglobin at Varying pH.

In order to calculate the base bound by oxyhemoglobin at varying pH ranges we utilize the fact that the buffer ratio $\frac{dB}{dpH}$ is constant over the physiological range, and has a value of 2.64. This means that as oxyhemoglobin combines with more alkali the pH increases at a constant proportion, the change being at the rate of 1 pH per 2.64 molecules of monovalent base (over the limited range, pH 7.2 to 7.5 at least.) Expressed in the usual form of a straight line equation, we have

$$B = [BHbO_2] = 2.64 [HbO_2] (pH - a) \quad (1)$$

where a is the pH at which the base bound would become zero, if the slope $\frac{dB}{dpH}$ continued unaltered to that point. If the $\frac{dB}{dpH}$ value remained absolutely constant up to that point, the latter would be the isoelectric point of oxyhemoglobin. As a matter of fact a is quite close to this point, but we are not at present certain how close. Consequently, it appears better to designate the extrapolated point merely as a , rather than by a symbol which might indicate a relation to the exact isoelectric point. The value of a , found by solving the above equation, is

$$a = pH - \frac{[BHbO_2]}{2.64 C} \quad (2)$$

From the data of Table VI we calculate in the last column of the table that the value of a lies between 6.58 and 6.62, the average being 6.61. However, the values in Table VI are uncorrected for the inactive hemoglobin. The average corrected value at pH 7.4 for $\frac{B}{C}$ viz. 2.15, gives $a = 6.585$.

Similarly we could estimate from points of known pH and B the value of b in the similar equation for reduced hemoglobin,

$$[\text{BHb}] = \beta_r [\text{Hb}] (\text{pH} - b) \quad (3)$$

From the data in Paper IV of this series (11), β_r is estimated as 2.45. From the average value of $\frac{[\text{BHb}]}{C}$ corrected for inactive hemoglobin, viz. 1.47 at pH 7.4, we obtain by calculation analogous to that of Equation 2, a value of 6.80 for b . This we use instead of the uncorrected average value of 6.79 given in Table VII.

$$[\text{BHb}] = 2.45 [\text{Hb}] (\text{pH} - 6.80) \quad (4)$$

Finally, by combining Equations 1 and 4 we obtain an expression relating the total base bound by hemoglobin to the pH and the per cent of oxygen saturation. Expressing the total base $[\text{BHbO}_2] + [\text{BHb}]$ as B, we have, by addition of Equations 1 and 4, Equation 5, which indicates the relationships of all three variables.

$$B = 2.64 [\text{HbO}_2] (\text{pH} - 6.585) + 2.45 [\text{Hb}] (\text{pH} - 6.80) \quad (5)$$

By substituting $C - [\text{HbO}_2]$ for $[\text{Hb}]$ we may solve Equation 5 in terms of $[\text{HbO}_2]$ as

$$B = 2.45 C (\text{pH} - 6.80) + (0.19 \text{ pH} - 0.72) [\text{HbO}_2] \quad (6)$$

Table VIII contains a comparison of observed and calculated values for $[\text{BHb}] + [\text{BHbO}_2]$, the "observed" being taken from the tables, the "calculated" being calculated by Equation 6. In making the calculations the inactive hemoglobin was calculated as HbO_2 . How much the observed deviation of about 1.0 mM. in No. 3 is due to error from this assumption is uncertain. The deviation in No. 4 is due to the fact, stated before, that almost certainly 3 mM. more alkali were added in the experiment than was intended.

In Equation 6 the term $2.45 C (\text{pH} - 6.80)$ represents the base that would be bound if all the hemoglobin were reduced, while the term $(0.19 \text{ pH} - 0.72) [\text{HbO}_2]$ represents the additional amount of base combined at the observed pH as the result of oxygenation of part of the hemoglobin, the coefficient $(0.19 \text{ pH} - 0.72)$ representing

$\frac{dB}{d[\text{HbO}_2]}$. This amount in equivalents of base added per mol of oxygen is, according to the results in the present paper, about 0.7 at pH 7.4. Calculated as $0.19 \text{ pH} - 0.7$, it is 0.67 at pH 7.30, 0.69 at pH 7.40, 0.71 at pH 7.50.

TABLE VIII.

Comparison of Observed and Calculated $[BHbO_2] + [BHb]$ Values.

Experiment No.	Table I.		Table II.		Table III.		Table IV.	
	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.
1	20.1	20.0	14.9	14.7	15.9	15.5	16.3	13.3
2	19.2	19.4	11.2	11.1	12.4	11.6	15.9	13.0
3	18.2	18.4	9.0	8.6	10.8	9.8	10.9	9.3
4	15.9	16.4			10.3	9.5	15.4	12.6
5	15.8	16.5	10.8	10.9	12.8	12.1	13.6	10.7
6	15.6	15.8	11.7	11.2	12.8	12.2	11.5	9.4
7	14.5	14.7	11.3	11.8	13.0	12.5	14.6	12.2
8	14.4	14.4	10.6	10.3	13.1	12.5		
9	13.7	13.9	10.8	10.6	10.8	10.0		
10	10.1	11.0			11.1	10.3		

Equation 5 and its derivatives are, of course, purely empirical, and hold only for the range over which β_o and β_r are constant. They afford, however, a convenient way of stating the observed relationships over at least the greater part of the physiological range of blood pH.

¹ As a matter of fact, Equation 6 was the first expression actually developed for empirically expressing the relationships of the three variables pH, B, and oxygen. It was developed by Hastings, who noted in charting Experiments, 4, 5, and 6 in the next paper (11) that $\frac{dB}{d[\text{HbO}_2]}$ increases with pH according to a linear function of the latter, and might therefore be expressed in the form $m \text{ pH} + n$.

An equation attempting to express the base bound at varying pH in terms of the mass law would have the form

$$B = C \left(\frac{[\text{HbO}_2]}{C} \frac{K_o}{K_o + [\text{H}^+]} + \frac{[\text{Hb}]}{C} \frac{K_r}{K_r + [\text{H}^+]} + \frac{K_s}{K_s + [\text{H}^+]} + \frac{K_4}{K_4 + [\text{H}^+]} + \dots \right)$$

with at least six terms, since as shown above there are at least five, and probably more, acid groups binding more or less base at normal blood pH in both oxygenated and reduced hemoglobin.

As L. J. Henderson has shown (6), assuming that changes in one acid group in hemoglobin are responsible for the difference in alkali-binding power between oxygenated and reduced hemoglobin, the group affected would have a relatively low dissociation constant, K_r , in reduced hemoglobin and a relatively high one, K_o , in oxygenated hemoglobin; and the value of K_o and K_r could be calculated from the difference in base bound by oxygenated and reduced hemoglobin at two different pH points. All our results are consistent with Henderson's assumption; and the values of K_o and K_r could be calculated from the difference in base bound by oxygenated and reduced hemoglobin at pH 7.2 and 7.4 estimated from our average data as expressed by equation 5 or 6. The change in $\frac{dB}{d[\text{HbO}_2]}$ with changing pH on which one must base calculations of K_o and K_r is slight, however, and it seems preferable to delay such calculation until experiments with hemoglobin solutions similar to Experiments 4, 5, and 6 with blood in the accompanying paper (11) are available, and a wider range of pH is covered so that the validity of the assumption can be tested by the constancy of results obtained with varying pairs of pH points.

The technique used in this paper may be of value in characterizing other proteins by determining the height and slope (buffer value) of their B, pH curves on the alkaline side of their isoelectric points. We have estimated above that the pH values determined are reproducible with a maximum error of 0.007 pH; the usual error need not exceed half as much. Such constancy appreciably exceeds that attainable by the electrometric method.

SUMMARY.

1. The alkali (Na) bound at pH 7.4 per gram molecule of re-crystallized horse oxyhemoglobin has been determined to be 2.15 ± 0.10 equivalents, that per gram molecule of reduced hemoglobin to be 1.47 ± 0.08 . The errors of ± 0.10 and ± 0.08 allowed for are about half due to the combined analytical errors connected with the technique, and half to the presence of 7 to 20 per cent of hemoglobin in a form incapable of binding labile oxygen in the preparations used. (In these calculations a gram molecule of hemoglobin has been taken as the amount combining with a gram molecule of oxygen.)

2. At pH 7.4, the change of 1 mol of reduced hemoglobin to oxyhemoglobin enables the hemoglobin to combine with 0.68 ± 0.10 equivalent of additional alkali.

3. For points intermediate between complete oxygenation and complete reduction, increase in base bound at constant pH is in simple direct proportion to increase in oxygen content. The $\frac{dB}{d[\text{HbO}_2]}$ ratio at a given pH is constant for all degrees of oxygenation. This fact accords with Henderson's theory (6).

4. The variation in base bound by oxyhemoglobin at varying pH is indicated by the molecular buffer value of $\beta_o = 2.64$. For each 0.1 pH increase, each molecule of oxyhemoglobin takes up 0.264 additional equivalents of base.

For reduced hemoglobin the molecular buffer value β_r is 2.45.

These buffer values are practically constant over the pH range 7.2 to 7.5.

5. The relationships summarized above are condensed into the empirical equation, valid over the pH range 7.2 to 7.5, and approximately so to pH 7.0 to 7.6:

$$B = 2.64 [\text{HbO}_2] (\text{pH} - 6.585) + 2.45 [\text{Hb}] (\text{pH} - 6.80)$$

The constants, 2.45 and 6.80, of the last term have been determined indirectly, and may be subject to slight revision when direct determinations are available.

6. The molecular buffer values, $\beta_o = 2.64$ and $\beta_r = 2.45$, indicate that at pH 7.2 to 7.5 at least five monovalent acid groups in the molecule share in the alkali combined with hemoglobin.

7. The constants above reported are for horse hemoglobin only. Preliminary experiments indicate that for dog hemoglobin the figures are different.

BIBLIOGRAPHY.

1. Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.
2. Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.
3. Van Slyke, D. D., *Physiol. Rev.*, 1921, i, 141.
4. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
5. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.
6. Henderson, L. J., *J. Biol. Chem.*, 1921, xlvi, 411.
7. Peters, J. P., Barr, D. P., and Rule, F. D., *J. Biol. Chem.*, 1920-21, xlv, 489.
8. Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.
9. Van Slyke, D. D., *Proc. Nat. Acad. Sc.*, 1921, vii, 229.
10. Bohr, C., *Skand. Arch. Physiol.*, 1905, xvii, 104.
11. Van Slyke, D. D., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.*, 1922, liv, 507.

STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN BLOOD.

IV. THE EFFECT OF OXYGENATION AND REDUCTION ON THE BICARBONATE CONTENT AND BUFFER VALUE OF BLOOD.

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(Received for publication, July 26, 1922.)

The experiments here presented were carried out in order—

1. To determine the buffer value of oxygenated and reduced blood, and the proportion of it for which hemoglobin is responsible.
2. To ascertain quantitatively the effect of change in combined oxygen at constant pH on the blood bicarbonate.
3. To ascertain the effect of changing pH on the $\frac{d[\text{BHCO}_3]}{d[\text{HbO}_2]}$ ratio.¹

We have used the same experimental methods employed with hemoglobin solution (1). The blood was saturated at 38° under known tensions of CO₂ and O₂, and the changes in base distribution were estimated from changes in the bicarbonate.

In the experiments with hemoglobin solutions (1) all changes in base present as BHCO₃ could be interpreted as changes in the opposite direction in base bound by hemoglobin, since carbonic acid and hemoglobin were the only acids present. Blood, however, also contains other buffers, chiefly in the form of plasma proteins and cell phosphate which also give off some bases to form bicarbonate when pH is lowered by increased CO₂ tension. Consequently, variations in [BHCO₃] accompanying changing pH due to changing CO₂ tension cannot be interpreted on blood as quantitative measures of opposite changes in [BHb] + [BHbO₂]. However, we may estimate the buffer value of oxyhemoglobin from its concentration and its molecular buffer values determined in the preceding paper (1), and by comparing the values

¹ The symbols used in the preceding paper (1) are employed.

thus estimated with the observed total buffer values we may ascertain the relative part played by oxyhemoglobin in the total buffer effect of the blood. The estimation from buffer constants obtained on hemoglobin solutions, of the buffer effect of hemoglobin in the blood involves the assumption that the confinement of hemoglobin within the red cells does not alter its buffer effect on the system. The approximate correctness of this assumption is shown by the results of Joffe and Poulton (2). Its absolute accuracy must be based on parallel experiments with laked and intact cells, which the temporary interruption of our work has forced us to leave to the future. The calculation of the relative part played by hemoglobin in the buffer effect of blood in Table VII is therefore presented with the reservation that slight corrections may be required because of the difference in pH between plasma and cell contents. Because of the relative constancy of the buffer value of hemoglobin over the pH range that the cell contents presumably cover, however, it does not appear that the correction can be considerable.

If we keep the pH of blood constant, however, by properly regulated CO_2 tension, and change only the degree of oxygenation, we appear justified in assuming that any changes in $[\text{BHCO}_3]$ that occur correspond quantitatively to changes in the opposite direction in base bound by hemoglobin. For hemoglobin is the only substance in the blood that combines with the oxygen, and can therefore conceivably undergo change in its chemical properties from oxygenation and reduction of the blood. Expressing these increases by the symbol Δ , with changing oxygen content at constant pH, $-\Delta[\text{BHCO}_3] = \Delta([\text{BHb}] + [\text{BHbO}_2])$.

EXPERIMENTAL.

The blood in volume from 250 to 400 cc. was drawn from the horse the night before each experiment. It was either oxalated or defibrinated, as described before (3), was chilled to nearly $0^\circ\text{C}.$, and was kept at that temperature until samples were removed for saturation the next morning. For the oxalation 0.2 per cent potassium oxalate plus 0.1 per cent of sodium fluoride was used (4).

The technique of saturation and analysis was identical with that used in the preceding paper (1) for hemoglobin solutions. For the blood CO_2 determinations 3 cc. samples were used.

The calculations of pH values were also performed as above (1), except that slightly different constants were required. For calculation of the pH values the value 6.15 for pK' was employed, instead of 6.18 as with hemoglobin solutions. The data on which the value 6.15 is based will be published later. For α_{CO_2} , Bohr's value of 0.511 for whole blood was employed.

Our experiments are divided into two groups.

In Experiments 1, 2, and 3 we have followed the procedure outlined for Experiments 1, 3, and 4 of the preceding paper (1). The slope of the $BHCO_3$, pH line has been determined on oxygenated blood, and used to extrapolate to a given pH the $[BHCO_3]$ values found in bloods in varying stages of oxygenation, and at pH's as near to the given pH as could be arranged by regulation of the CO_2 and O_2 tensions. By these experiments we obtained data on the buffer value of oxygenated blood, on the effect of oxygenation and reduction on $[BHCO_3]$ at constant pH, and on the proportionality between this effect and the degree of oxygenation. The results are given in Tables I, II, and III, and in Figs. 1a, 1b, 2a, 2b, 3a, and 3b.

In order to obtain data also on the buffer value of reduced blood, and on the total effect of reduction and oxygenation at varying pH, we have carried out Experiments 4, 5, and 6. In each of these a number of $[BHCO_3]$ values were obtained with blood approximately completely oxygenated, $p_{O_2} = 140$ mm. at varying pH, and a number of other $[BHCO_3]$ values were obtained on blood as near as possible to complete reduction. The $[BHCO_3]$ points for both oxygenated and reduced bloods when plotted against pH made absolutely straight lines within the pH range of the experiments, 7.1 to 7.5.

The results of Experiments 4, 5, and 6 are given in Tables IVc, IVb, Va, Vb, VIa, and VIb, and in Figs. 4, 5, and 6. In tables IVb, Vb, and VIb, the values for $\frac{d[BHCO_3]}{d[HbO_2]}$ may be taken, for reasons

discussed above, to represent negative values of $\frac{dB}{d[HbO_2]}$ in the sense used in the preceding paper, B indicating the total base bound by hemoglobin as $[BHb] + [BHbO_2]$ (1).

EXPLANATION OF TABLES AND FIGURES.

In all except Tables IV*b*, V*b*, and VI*b* the symbols used are the same as in the tables of the preceding paper. In Tables IV*b*, V*b*, and VI*b*, we have used $[\text{BHCO}_3]_r$ for the $[\text{BHCO}_3]$ of the reduced blood; $[\text{BHCO}_3]_o$ for the $[\text{BHCO}_3]$ of the oxygenated blood; $\Delta[\text{BHCO}_3] = [\text{BHCO}_3]_r - [\text{BHCO}_3]_o$. The ratio $\frac{\Delta[\text{BHCO}_3]}{\Delta[\text{HbO}_2]}$ is always negative since at constant pH, oxygen and bicarbonate contents move in opposite directions.

The charts are designed like those in the preceding paper, except that in the present one the $[\text{BHCO}_3]$ values are plotted as ordinates, instead of the $[\text{BHb}] + [\text{BHbO}_2]$ values. Consequently, since the $[\text{BHCO}_3]$ varies in the opposite direction from the $[\text{BHb}] + [\text{BHbO}_2]$ in the present charts the lines slope in the opposite direction from those in the preceding paper, and the oxygenated line is at the bottom of a series instead of at the top.

TABLE I.

Horse Blood No. 1.

Anticoagulant Oxalate + NaF.
Total oxygen capacity 7.30 mm.

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	Total [CO ₂]	[BHCO ₃]	pH	[BHCO ₃] at pH 7.30.
	mm.	mm.	mm.	mm.	mm.	mm.		mm.
1	(140)	7.39	7.21	33.2	16.05	15.05	7.329	15.75
2	(140)	7.36	7.18	47.1	18.64	17.23	7.236	15.75
3	(140)	7.38	7.20	61.7	20.90	19.05	7.162	15.75
4	49.30	5.75	5.68	44.4	18.55	17.22	7.265	16.40
5	34.50	4.49	4.45	44.4	19.05	17.72	7.274	17.10
6	32.70	4.29	4.25	44.2	19.15	17.82	7.278	17.28
7	21.60	2.26	2.24	46.6	20.35	18.95	7.282	18.55
8	2.9	0.32	0.32	51.4	21.76	20.22	7.268	19.47
9	142.0	7.38	7.20	40.6	17.52	16.30	7.276	15.75

DISCUSSION OF RESULTS.

The Buffer Value of Oxygenated and Reduced Blood and the Proportion of It for Which Hemoglobin Is Responsible.

The buffer values for oxygenated blood, in mm. terms, are given in Table VII for the five bloods on which they were determined.

FIG. 1a. From the line through the oxygenated points 1, 2, 3, and 9, $d[\text{BHCO}_2]$ is calculated as -23.7 .

Intersections of lines through the other points with the pH 7.3 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table I from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH line are given.

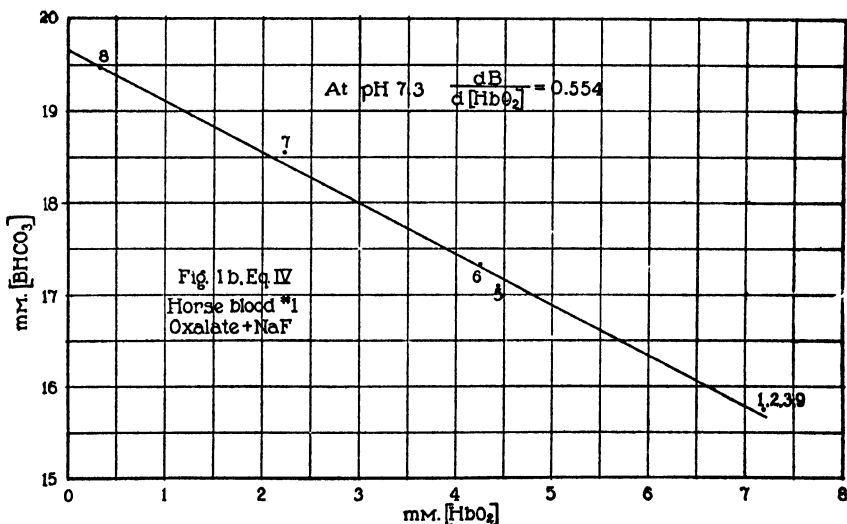
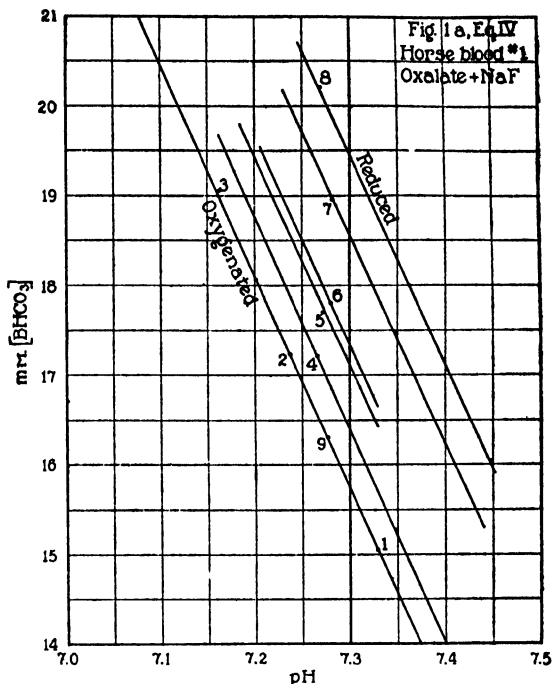


FIG. 1b. Amount of base displaced from BHCO_2 at pH 7.3 compared with amounts of oxygen bound. Data from Table I and Fig. 1a.

From the slope of the line, $\frac{d[\text{BHCO}_2]}{d[\text{HbO}_2]}$ is calculated as -0.554 .

(On No. 3 the buffer slope was not determined, but was estimated for purposes of extrapolation from the hemoglobin content.) The value of the buffers other than BHCO_3 was estimated as $\frac{dB}{dpH} = -\frac{d[\text{BHCO}_3]}{dpH}$ since increase in base bound as BHCO_3 is equal to loss of base by other buffers. The other calculations in the table are self-explanatory. It appears that from 73 to 79 per cent of the total buffer effect of the oxygenated horse blood was due to oxyhemoglobin. The average buffer effect of buffers other than bicarbonate, in mM. terms, was $\frac{dB}{dpH} = 22.6$, of which 19.0 were due to hemoglobin,

TABLE II.

Horse Blood No. 2.

Anticoagulant.....Oxalate and NaF.

Total oxygen capacity.....6.80 mM.

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	Total [CO ₂]	[BHCO ₃]	pH	[BHCO ₃] at pH 7.275.
	mM.	mM.	mM.	mM.	mM.	mM.		mM.
1	143.3	6.93	6.75	33.0	16.79	15.80	7.353	17.35
2	142.8	6.95	6.77	42.3	18.56	17.29	7.274	17.35
3	(143.0)	6.86	6.68	62.2	21.63	19.57	7.170	17.35
4	44.2	5.26	5.20	44.7	19.47	18.13	7.271	18.07
5	31.5	3.85	3.81	44.7	20.06	18.72	7.298	19.26
6	27.5	3.03	2.99	45.6	20.40	19.03	7.293	19.42
7	21.0	1.90	1.87	49.5	21.45	19.96	7.278	20.04
8	1.5	0.14	0.14	48.7	22.13	20.67	7.291	20.98
9	141.4			42.4	18.51	17.24	7.282	17.35

leaving 3.6 to buffers other than hemoglobin and bicarbonate. The bicarbonate contributed an average $\frac{dB}{dpH}$ value of 1.8, making the total 24.4.

In percentages of the total buffer effect, the average values for all five bloods are 78 per cent for hemoglobin, 7.4 per cent for bicarbonate, and 14.6 for all other buffers.

The results obtained with reduced blood in Experiments 4, 5, and 6 are given in Table VIII. The effects of buffers other than hemoglobin and BHCO_3 , are estimated in Column d of Table VIII from the oxygenated samples by subtracting the calculated buffer effect of

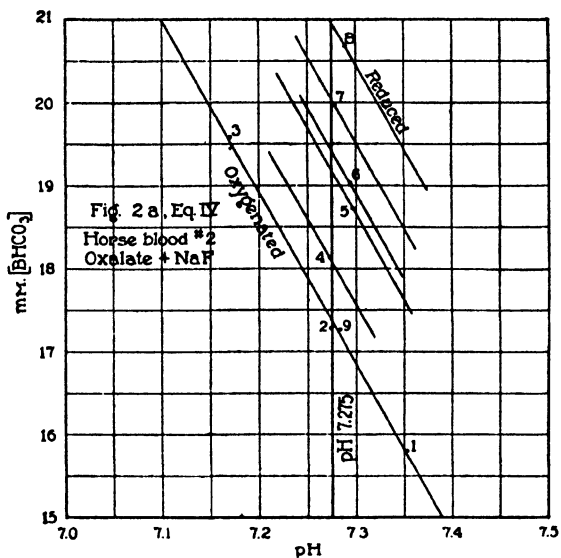


FIG. 2a. From the line through the oxygenated points 1, 2, 3, and 9, $\frac{d[\text{BHCO}_3]}{dpH}$ is calculated as -20.6 .

Intersections of lines through the other points with the pH 7.275 line indicate effects of varying degrees of reduction in lowering the amount of base bound by the hemoglobin.

The numbers near the points indicate the data in Table II from which they are taken. In the last column of the table the amounts of base indicated by the intersections on the pH line are given.

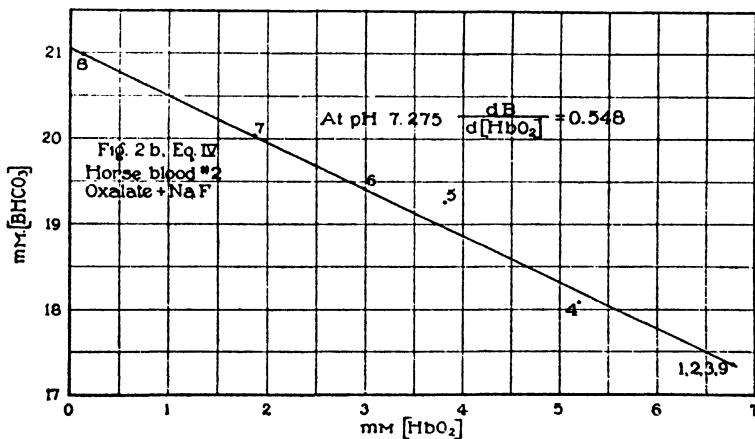


FIG. 2b. Amounts of base displaced from BHCO_3 at pH 7.275 compared with amounts of oxygen bound. Data from Table II and Fig. 2a.

From the slope of the line $\frac{d[\text{BHCO}_3]}{d[\text{HbO}_2]}$ is calculated as -0.548 .

the oxyhemoglobin from the total. In the reduced bloods it is assumed that the effects of the buffers other than BHCO_3 and hemoglobin are the same as in oxygenated. The buffer value of the reduced hemoglobin is accordingly estimated by subtracting the values in Column d from those in Column e. From the $\frac{dB}{d\text{pH}}$ values found, the molecular buffer value is calculated in Column g as $\beta_r = \frac{dB}{CdpH}$.

From comparison in Tables VII and VIII of the average values for those experiments in which both oxygenated and reduced bloods were examined, the shift of buffer values resulting from reduction of

TABLE III.

Horse Blood No. 3.

Anticoagulant Defibrinated.
Total oxygen capacity 6.68 mm.

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	Total [CO ₂]	[BHCO ₃]	pH	[BHCO ₃] at pH 7.24.
	mm.	mm.	mm.	mm.	mm.	mm.		mm.
1	143.5	6.75	6.56	41.9	16.65	15.39	7.238	15.38
2	37.6	4.30	4.25	46.0	18.14	16.76	7.235	16.63
3	19.9	1.90	1.87	47.3	19.08	17.66	7.245	17.74
4	4.0	0.22	0.22	48.7	19.91	18.45	7.251	18.67
5	135.0	6.69	6.49	43.4	16.80	15.50	7.226	15.25
6	29.70	3.21	3.17	45.0	18.13	16.78	7.244	16.85
7	20.80	2.00	1.97	46.2	18.85	17.46	7.250	17.66
8	3.5	0.19	0.19	48.7	19.93	18.47	7.252	18.73

oxygenated blood at constant pH becomes apparent. The hemoglobin decreases in both percentage and absolute buffer effect, because of the chemical change it undergoes; the bicarbonate increases in both percentage and total buffer effect, because of the increase in $[\text{BHCO}_3]$, and its gain nearly balances the loss of the hemoglobin.

The Effect of Change in Combined Oxygen at Constant pH on the Blood Bicarbonate.

The values for the $\frac{d[\text{BHCO}_3]}{d[\text{HbO}_2]}$ ratios obtained at 7.30, or the nearest point to 7.30, covered by observation, are given in Table IX.

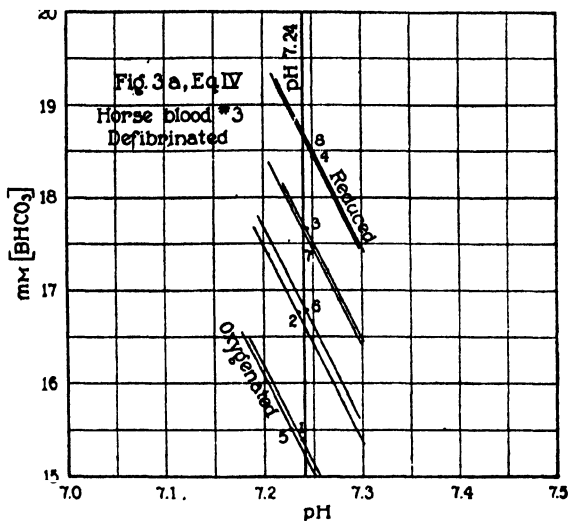


FIG. 3a. The slope of the lines is calculated as $\frac{CB_o}{0.85}$ from the hemoglobin content.

Intersections of lines through the other points with the pH 7.24 line indicate effects of varying degrees of reduction in lowering the amount of the base bound by the hemoglobin.

The numbers near the points indicate the data in Table III from which they are taken. In the last column of the Table the amounts of base indicated by the intersections on the pH 7.24 line are given.

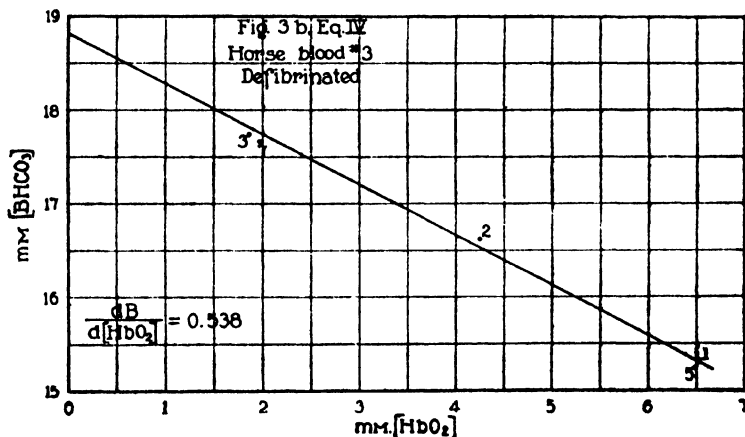


FIG. 3b. Amounts of base displaced from $BHCO_3$ at pH 7.24 compared with amounts of oxygen bound. Data from Table III and Fig. 3a.

From the slope of the line, $\frac{d[BHCO_3]}{d[HbO_2]}$ is calculated as -0.538 .

TABLE IVa.

Horse Blood No. 4.

Anticoagulant.....Oxalate+NaF.
 Total oxygen capacity.....7.16 mm.
 Technique.....Double tonometer.
 Double equilibration.

No.	P _{O₂}	Total [O ₂]	[HbO ₂]	P _{CO₂}	Total [CO ₂]	[BHCO ₂]	pH
	mm.	mm.	mm.	mm.	mm.	mm.	
1	136.9	7.30	7.12	54.7	19.55	17.91	7.188
2	136.2	7.27	7.09	53.8	19.35	17.74	7.191
3	128.2			31.0	15.25	14.32	7.338
4a	130.3	7.32	7.15	24.2	13.57	12.84	7.398
4b	130.6	7.31	7.14	24.2	13.50	12.77	7.395
Average.....			7.13				
5	2.6	0.22	0.22	63.0	23.27	21.38	7.203
6	3.3	0.25	0.25	46.0	20.66	19.28	7.295
7	3.5	0.31	0.31	34.2	18.44	17.41	7.379
8	3.6	0.33	0.33	25.8	16.43	15.66	7.456
Average.....			0.28				

TABLE IVb.

Horse Blood No. 4. Data from Fig. 4.

pH	[BHCO ₂] _R	[BHCO ₂] _O	Δ[BHCO ₂]	$-\frac{\Delta[\text{BHCO}_2]^*}{\Delta[\text{HbO}_2]}$
	mm.	mm.	mm.	
7.1	23.70	20.00	3.70	0.540
7.2	21.46	17.59	3.87	0.565
7.3	19.20	15.16	4.04	0.589
7.4	16.96	12.75	4.21	0.615
7.5	14.70	10.32	4.38	0.639

$$*\Delta[\text{HbO}_2] = 0.28 - 7.13 = -6.85$$

The assumption appears justified, as stated above, that, at constant pH, changes in oxygenation affect the amounts of base bound only by carbonic acid and by hemoglobin, whatever base is detached from combination with hemoglobin being represented by increase in [BHCO₂], and *vice versa*. If, therefore, no factors in blood alter the pK'_R and pK'_O values of the acid group or groups of which the base-

binding power is affected by oxygen changes, we should expect at a given pH the $\frac{dB}{d[\text{HbO}_2]}$ ratio determined in sodium hemoglobinate solutions to have the same value as the $\frac{d[\text{BHCO}_2]}{d[\text{HbO}_2]}$ ratio determined in blood, except for change in sign.

However, at pH 7.30 the results of sodium hemoglobinate solutions (1) have indicated a value of 0.67 for the $\frac{dB}{d[\text{HbO}_2]}$ ratio. This is

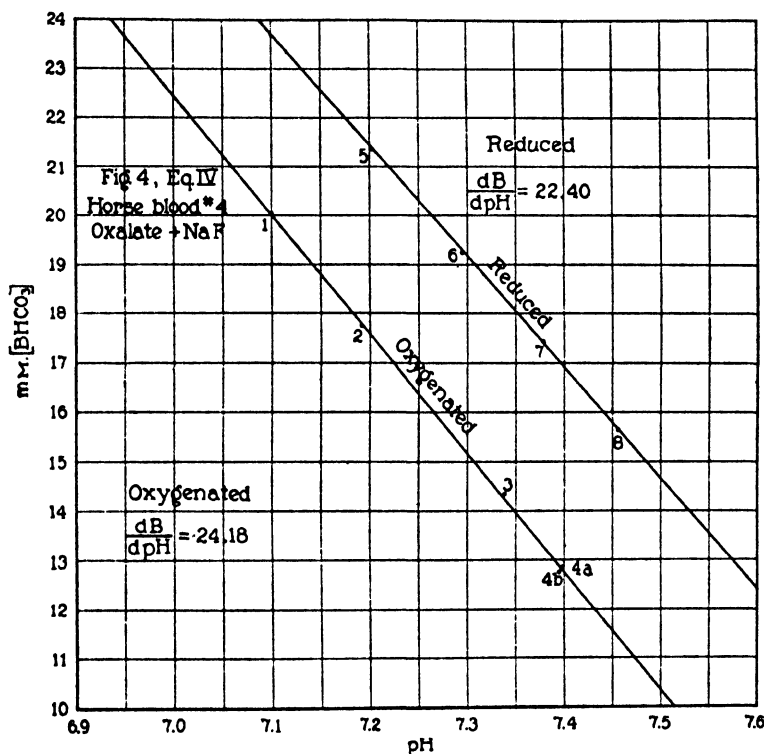


FIG. 4. From the line through the oxygenated points 1, 2, 3, 4a, and 4b, $\frac{d[\text{BHCO}_2]}{dpH}$ for the oxygenated blood is calculated as -24.18 . From the line through points 5, 6, 7, and 8, $\frac{d[\text{BHCO}_2]}{dpH}$ for reduced blood is calculated as -22.40 .

The numbers near the points indicate the data in Table IVa from which they are taken.

TABLE Va.

Horse Blood No. 5.

Anticoagulant Oxalate + NaF.
 Total oxygen capacity 6.95 mm.

No.	P _{O₂} *	Total [O ₂].	[HbO ₂]	P _{CO₂}	Total [CO ₂].	[BHCO ₂]	pH
	mm.	mm.	mm.	mm.	mm.	mm.	
1	(140)	7.13	6.95	52.5	19.28	17.70	7.200
2	(140)			36.3	16.50	15.41	7.301
3	(140)	7.11	6.93	26.5	14.38	13.58	7.382
4	(140)	7.12	6.94	19.9	12.65	12.05	7.455
5	(0)	0.40	0.40	60.8	22.63	20.81	7.207
6	(0)	0.28	0.28	44.5	20.19	18.85	7.299
7	(0)	0.37	0.37	31.3	17.63	16.69	7.400
8	(0)	0.16	0.16	22.5	15.48	14.80	7.491
9	(140)	6.98	6.80	63.1	20.68	18.79	7.148
10	(140)	7.19	7.01	46.2	18.27	16.88	7.236
11	(140)	7.15	6.97	31.3	15.50	14.56	7.341
12	(140)	7.06	6.85	21.5	12.95	12.30	7.431
13	(0)	0.21	0.21	72.1	24.03	21.87	7.155
14	(0)	0.17	0.17	51.9	21.29	19.73	7.253
15	(0)	0.30	0.30	35.9	18.57	17.49	7.361

* P_{O₂} figures in this table represent oxygen tension of gas placed in tonometers for second saturation, not the final tension determined by analysis.

TABLE Vb.

Horse Blood No. 5. Data from Fig. 5.

pH	[BHCO ₂] ₀	[BHCO ₂] _R	Δ[BHCO ₂]	- $\frac{\Delta[\text{BHCO}_2]^*}{\Delta[\text{HbO}_2]}$
	mm.	mm.	mm.	
7.1	19.88	23.00	3.12	0.469
7.2	17.65	20.90	3.25	0.489
7.3	15.42	18.80	3.38	0.508
7.4	13.20	16.70	3.50	0.526
7.5	10.97	14.60	3.63	0.546

* Δ[HbO₂] = - 6.65

higher than any of the $\frac{dB}{d[\text{HbO}_2]}$ values obtained in blood, and it appears probable that the difference is outside the limits of experimental error.

Also it appears that the difference in the value of $\frac{dB}{d[\text{HbO}_2]}$ as determined at pH 7.3, in Bloods 4 and 5, respectively, is outside the

limits of experimental error. The experiments on these two bloods were performed near the end of our work when the accuracy of our technique was at its best, and a number of points on oxygenated blood and blood at a uniform degree of nearly complete reduction were obtained.

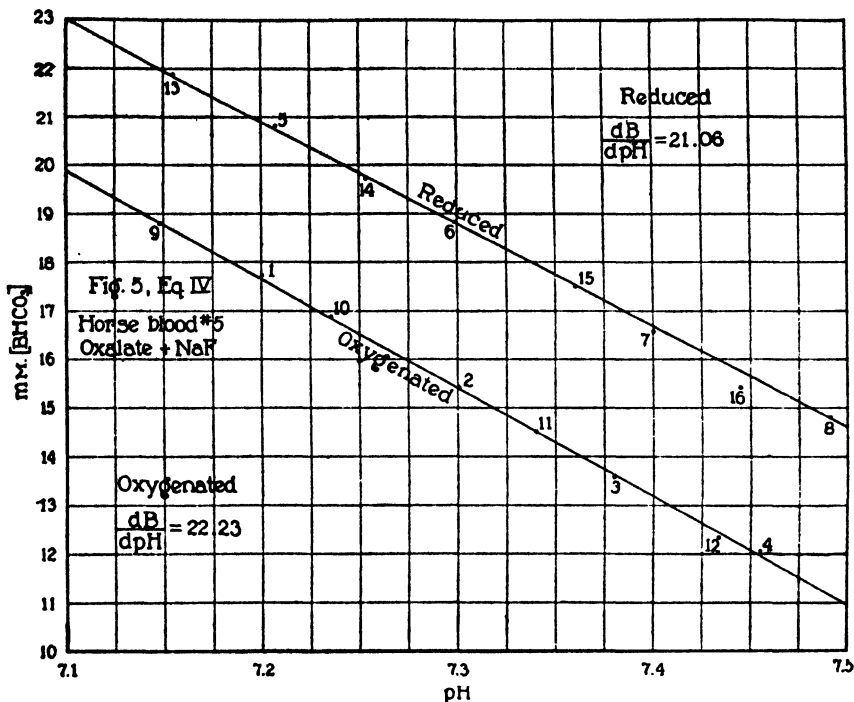


FIG. 5. From the line through the oxygenated points, $\frac{d[BHCO_3]}{dpH}$ is calculated as -22.23 . From the line through the reduced points $\frac{d[BHCO_3]}{dpH}$ for reduced blood is calculated as -21.06 .

The numbers near the points indicate the data in Table Va from which they are taken.

Both bloods were oxalated and otherwise treated in an identical manner. They were also drawn from the same horse at times only 3 days apart. Yet the $\frac{dB}{d[HbO_2]}$ ratio in Experiment 4 is 0.589, and in Experiment 5 is 0.508.

There appear to be factors present in the blood which depress the $\frac{dB}{d[\text{HbO}_2]}$ ratio (B representing base bound by hemoglobin) below the value that it has in solution of sodium hemoglobinate and bicarbonate, and which depress it to a variable extent. One of these factors is

TABLE VIA.

Horse Blood No. 6.

Anticoagulant.....Defibrinated.
Total oxygen capacity.....7.81 mm.

No.	P_{O_2} *	Total $[\text{O}_2]$	$[\text{HbO}_2]$	P_{CO_2}	Total $[\text{CO}_2]$	$[\text{BHCO}_2]$	pH
	mm.	mm.	mm.	mm.	mm.	mm.	
1	(140)	7.86	7.68	52.1	18.32	16.76	7.180
2	(140)	7.81	7.63	37.2	15.66	14.54	7.265
3	(140)	7.97	7.79	25.9	13.21	12.43	7.354
4	(140)	7.91	7.73	18.7	11.23	10.67	7.429
5	(0)	0.78	0.78	59.8	21.57	19.78	7.193
6	(0)	1.04	1.04	43.6	18.94	17.63	7.279
7	(0)	0.98	0.98	31.2	16.52	15.58	7.371
8	(0)	1.01	1.01	22.8	14.38	13.70	7.452

* The p_{O_2} figures in this table represent oxygen tension of gas placed in tonometers for second saturation, not final tension determined by analysis.

TABLE VIb.

Horse Blood No. 6. Data from Fig. 6.

pH	$[\text{BHCO}_2]_0$	$[\text{BHCO}_2]_R$	$\Delta[\text{BHCO}_2]$	$-\frac{\Delta[\text{BHCO}_2]^*}{\Delta[\text{HbO}_2]}$
	mm.	mm.	mm.	
7.2	16.25	19.54	3.29	0.487
7.3	13.80	17.20	3.40	0.503
7.4	11.37	14.90	3.53	0.522
7.5	8.91	12.59	3.68	0.544

* $\Delta[\text{HbO}_2] = -6.76$

probably the difference in pH between cells and plasma. The pH values that we have estimated in the blood are those of the plasma. The pH values in the cells are probably lower; and, as will be brought out below, depression of the pH depresses the $\frac{dB}{d[\text{HbO}_2]}$ ratio.

It may be that the difference in concentration of salts in the blood, as compared with our sodium hemoglobin solutions, also affects somewhat the pK' values of the acid group or groups in the hemoglobin molecule which are altered by oxygenation. The question as to whether these two factors are sufficient to account for the observed variations in the ratio must await the accumulation of more data.

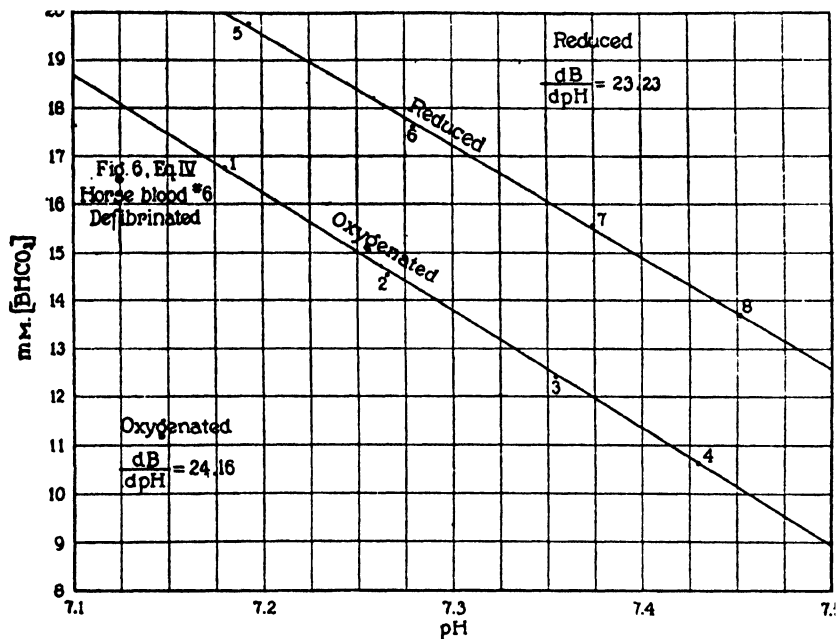


FIG. 6. From the line through the oxygenated points 1, 2, 3, and 4, $\frac{d[BHCO_3]}{dpH}$ is calculated as -24.16 . From the line through the reduced points 5, 6, 7, and 8, $\frac{d[BHCO_3]}{dpH}$ for reduced blood is calculated as -23.23 .

The numbers near the points indicate the data in Table VIa from which they are taken.

The Effect of Changing pH on the $\frac{d[BHCO_3]}{d[HbO_2]}$ Ratio.

From Tables IVb, Vb, and VIb it will be seen that the value of the ratio $-\frac{\Delta[BHCO_3]}{\Delta[HbO_2]}$ increases with increasing pH. The rates of

TABLE VII.
The Relative Part Played by Hemoglobin in the Total Buffer Effect of Oxygenated Blood at pH 7.35.

Blood No.	Condition.	Total buffer value of oxygenated blood exclusive of bicarbonate.*	[BHC ₃ O ₃] at pH 7.35	Buffer value of [BHC ₃ O ₃] at pH 7.35 = 0.127† [BHC ₃ O ₃]	Total buffer value of blood a + c.	Hemoglobin content [HbO ₂].	Estimated oxyhemo- globin buffer effect = 2.64 c.	Proportion of total buffer effect due to		
		a	b	c	d	e	f	Oxyhemo- globin.	[BHC ₃ O ₃]	Other buffers.
		dB dpH	mm.	dB dpH	dB dpH	mm.		per cent	per cent	per cent
1	Oxalated.	23.72	15.75	2.00	25.75	7.30	19.27	74.8	7.8	17.4
2	"	20.64	15.80	2.01	22.65	6.80	17.94	79.2	8.9	11.9
4	"	24.18	14.00	1.78	25.96	7.16	18.90	72.8	6.9	20.3
5	"	22.23	14.30	1.83	24.06	6.95	18.35	76.3	7.6	16.1
6	Defibrinated.	24.16	12.60	1.60	25.76	7.72	20.36	79.0	6.2	14.8
Average of Nos. 4, 5, and 6.....					25.26			76.0	6.9	17.1

* The buffer values are expressed in mm. terms of B.

† pK' for BHC₃O₃ being 6.15. pH - pK' = 7.35 - 6.15 = 1.20. For pH - pK' = 1.20. Fig. 9 of a former paper on buffers (5) shows 22 per cent of maximum buffer value is exerted. The maximum is 0.575 C, where C is the concentration of the buffer. 0.575 × 0.22 × C = 0.127 C.

TABLE VIII.
Buffer Values in Reduced Blood, and Estimation of β_2 in Experiments 4, 5, and 6.

Blood No.	Total hemoglobin content from O ₂ capacity.	Values of $\frac{dB(\text{mm.})}{dpH}$ exclusive of bicarbonate.						Molecular buffer value of reduced Hb. $\beta_2 = \frac{f}{a}$	[BHC ₃] at pH 7.35.	Buffer† value of [BHC ₃] at pH 7.35.	Total buffer value of reduced blood.	Proportion of total effect due to		
		For oxy-generated blood.		For* [HbO ₂] in oxy-generated blood. $= a \times \beta_0$	For buffers other than [HbO ₂] $= b - c$	For reduced blood.	Reduced hemoglobin. $= e - d$					Reduced hemoglobin.	[BHC ₃].	Other buffers.
		Observed.	c											
		e	b										ϵ	
	mm.							mm.	$\frac{dB}{dpH}$	$\frac{dB}{dpH}$	$\frac{dB}{dpH}$	per cent	per cent	per cent
4	7.16	24.18	18.90	5.28	22.40	17.12	2.40	18.08	2.30	24.70	69.3	9.3	21.4	
5	6.95	22.23	18.35	3.88	21.06	17.18	2.47	17.75	2.25	23.31	73.7	9.6	16.7	
6	7.72	24.16	20.36	3.80	23.23	19.43	2.51‡	16.02	2.04	25.27	77.0	8.0	15.0	
Average.....							2.45			24.43	73.3	9.0	17.7	

* β_0 = molecular buffer value of oxyhemoglobin = 2.64 from previous paper.

† See dagger (†) foot-note to Table VII.

‡ This solution was only 88 per cent reduced. If it were completely reduced the value would presumably be 2.49 instead of 2.51. The figure 2.49 is used in calculating the average.

increase in the ratio per unit increase in pH are 0.248, 0.192, and 0.190 in Experiments 4, 5, and 6, respectively, or about 0.02 for each 0.1 change in pH over the range pH 7.5 to 7.1.

From the curves of Figs, 4, 5, and 6 it is possible by a method of calculation already outlined by L. J. Henderson (4) to estimate the values K'_r and K'_o of the monovalent acid group in the hemoglobin molecule that has its dissociation constant changed from K_r to K when reduced hemoglobin is oxygenated, the assumption being made that only one such group is so changed. In view of the appreciable differences observed between different bloods, however, and between blood and solutions of recrystallized hemoglobin it appears preferable to let the publication of such calculations await further data.

TABLE IX.

Decrease in $[BHCO_3]$ per Gram Molecule of Oxygen Combined at Constant pH.

Experiment No.	pH	Treatment of blood.	Mols decrease in $[BHCO_3]$ per mol increase in $[HbO_2]$
1	7.30	Oxalate + NaF.	0.554
2	7.275	" + "	0.548
3	7.24	Defibrinated.	0.538
4	7.30	Oxalate + NaF.	0.589
5	7.30	" + "	0.508
6	7.30	Defibrinated.	0.503

SUMMARY.

1. The average buffer value at pH 7.2 to 7.5 of three samples of oxygenated blood from the same horse was found to be $\frac{dB(\text{mm.})}{dpH} = 25.3$ the average value for the same bloods in the reduced condition being 24.4.
2. The buffer values of each blood, the degree of oxygenation being constant, were constant over the pH range 7.2 to 7.5.
3. In the oxygenated blood the hemoglobin was calculated to be responsible for an average of 76.0 per cent of the total buffer value, bicarbonate for 6.9 per cent. In reduced hemoglobin the figures were 73.3 and 9.0, respectively.
4. The difference in buffer value between oxygenated and reduced blood is attributable to a loss of buffer value at this pH when oxy-

hemoglobin is reduced. The consequent loss to the total buffer value of the blood is only partially compensated by the increase in $[\text{BHCO}_3]$ that accompanies the deoxygenation.

5. From the effect of oxygenation and reduction on the buffer value of blood it is estimated that if the molecular buffer value of oxyhemoglobin is 2.64, as found in the preceding paper, the molecular buffer value of reduced hemoglobin is 2.45.

6. At pH 7.3 the decrease in $[\text{BHCO}_3]$ per added molecule of oxygen combined with the hemoglobin was found to vary in six bloods from 0.50 to 0.59, indicating that a gram molecule of oxygenated hemoglobin at this pH binds 0.50 to 0.59 more gram equivalents of alkali than does reduced hemoglobin. The variation between 0.50 and 0.59 is probably outside the limit of error in these experiments, and is due to variable factors not yet ascertained in the blood. The ratios observed in blood are lower than those observed in solutions containing only recrystallized hemoglobin and sodium bicarbonate.

7. At a given pH, with varying degrees of oxygenation, the displacement of base from combination with bicarbonate is in simple direct proportion to the amount of oxygen combined. This constancy of the $\frac{dB}{d[\text{HbO}_2]}$ ratio was also found with hemoglobin solutions (1).

8. Increase of pH over the range 7.2 to 7.5 increases the amount of alkali in a given blood displaced from combination with bicarbonate (added to hemoglobin) by 1 molecule of oxygen, the increase being at the rate of, approximately, 0.02 equivalent of alkali per 0.1 pH rise.

9. No quantitative differences were detected between defibrinated blood and blood in which coagulation had been prevented by addition of 0.2 per cent of potassium oxalate and 0.1 per cent of sodium fluoride.

BIBLIOGRAPHY.

1. Van Slyke, D. D., Hastings, A. B., Heidelberger, M., and Neill, J. M., *J. Biol. Chem.*, 1922, liv, 481.
2. Joffe, J., and Poulton, E. P., *J. Physiol.*, 1920, liv, 129.
3. Evans, C. L., *J. Physiol.*, 1922, lvi, 146.
4. Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.
5. Van Slyke, D. D., *J. Biol. Chem.*, 1922, lii, 525.

THE ACTION OF SODIUM SALICYLATE UPON THE FORMATION OF IMMUNE BODIES.

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(Received for publication, July 7, 1922.)

INTRODUCTION.

This study is one of a series to determine the action of the salicylates upon some of the manifestations of infection in the animal body. From a pharmacological view-point it is well established that the salicylates have an antipyretic and analgesic action in patients when administered in therapeutic doses. The analgesic action varies in different individuals and is apparently more powerful with some of the salicylate compounds than with others. This effect is obtained in many conditions, both in the general toxic state seen in most infections, and also in neuralgias and myalgias when indefinite pain is the chief symptom, and fever and other general manifestations of infectious intoxication are usually absent.

The antipyretic action is due to an increased loss of body heat and, as shown by Barbour (1), is evidenced almost exclusively by individuals who are suffering from infection; normal persons show little if any antipyretic effect after taking fairly large doses of the drug. Afebrile patients suffering from infections, on the other hand, show an increased heat loss after receiving comparatively small doses of the drug. It seems as though the heat-regulating center of this class of individuals is in a different state of sensitiveness to salicylates than is that of normal persons.

A third action of salicylates demonstrated almost exclusively in patients with the polyarthritis of rheumatic fever is that of an antiphlogistic agent. Following the administration of the drug in amounts just under the toxic dose, a most striking decrease of local heat, redness, and swelling, as well as of pain and tenderness in the

involved joints is usually observed; and, in addition, the tendency for the inflammation to spread to other joints is arrested. Patients suffering from gonorrheal arthritis or other joint infections in which bacteria can be demonstrated in the arthritic fluid are not benefited in this striking manner by salicylates. So often has this observation been repeated that the therapeutic test is frequently applied to differentiate the acute polyarthritides of rheumatic fever from that due to other causes. It seems fairly well established, on the other hand, that the duration of the general infection in rheumatic fever is not materially shortened by the drug, and that cardiac complications often arise even though the patients are taking amounts sufficient to keep their temperatures almost normal and to prevent arthritis recurring. From clinical observation alone it would seem, therefore, that the drug either lowered the infectivity of the etiologic agent of rheumatic fever by a direct action upon the virus or increased the ability of the body to react against the infection.

It is well known that salicylic acid and sodium salicylate have an antiseptic action *in vitro*; this action is much stronger with free salicylic acid than with its salts. It is, moreover, impossible for the free acid to occur in the tissues or body fluids in sufficient concentration to exert any marked bactericidal action. A bacteriostatic action must be considered; the drug may exist in sufficient concentration or in a state to inhibit the growth of the infectious agent, even though the virus is not completely killed. A study of this problem is now under way.

It is conceivable that the salicylates may increase the resisting power of the animal body by stimulating the formation of immune bodies; but in so far as we are able to determine very little attention has been given to this possibility.

Jacoby and Schütze (2) studied the opsonin and bacteriotropin content of the serum of normal and immune rabbits; the serum was obtained both before and 8 hours after the animals received a single dose of 2 gm. of sodium salicylate by stomach tube. *Staphylococci*, *B. coli*, and *B. typhosus* were used in the tests. The authors state that five out of twelve rabbits showed a distinct increase of immune bodies following salicylate treatment. Unfortunately, the details of the experiments are not given, so it is impossible to tell whether the opsonins or the bacteriotropins were increased the more.

EXPERIMENTAL.

In view of the fact that no definite etiologic agent has been demonstrated for rheumatic fever, it was thought advisable in our studies to use various members of the coccus group of bacteria as antigens to stimulate the formation of antibodies in rabbits, and to compare the amount of antibody formation in immunized animals receiving sodium salicylate with that of animals similarly immunized but untreated with salicylates. In some of the experiments living bacteria were inoculated into the rabbits, in others killed bacteria in the form of vaccines were employed. In all instances the antigens were injected intravenously. In addition, three experiments were performed to determine the effect of salicylates upon hemolysin formation.

The rabbits received the sodium salicylate in the form of a 2.5 or 5 per cent solution twice daily by stomach tube. In the earlier experiments this was combined with an equal amount of sodium bicarbonate; but later it was found that the salicylate was well tolerated without the bicarbonate so the latter was omitted. In most instances the daily dose of sodium salicylate was from 0.16 to 0.2 gm. per kilo of body weight; the weight of the animals was determined from an average computed by repeated weighings for several days before the actual start of the experiments. Subsequent change in the weight of the rabbits was not used as an indication for altering the dose.

The rabbits were bled before beginning inoculation and twice a week subsequently. The separated serum was kept in the ice box, and the sera obtained at several different dates were tested at one time against the homologous antigens. This precaution was taken so that the results might be as comparable as possible. In all except the first two or three experiments a single standardized pipette was used to dilute all of the sera in one series of tests; this pipette was washed five or six times with saline solution after making each dilution. In doing comparative work with dilutions of different sera this precaution was necessary, for the ordinary pipettes were found to vary from 10 to 20 per cent. After recording the strength of reaction in the ordinary way by a series of plus marks the antibody content was computed from an interpolation table and curve. In this way the strength of reaction can be recorded by means of curves or figures and the comparison made easier than when the reactions are merely recorded by a certain number of pluses.

In some of the earlier experiments the temperature of the rabbits was charted twice daily; subsequently this procedure was discontinued as it was found that the information obtained was of little value in interpreting the immunity results.

Influence of Salicylates on the Formation of Complement-Fixing Antibodies.

In the first three experiments the formation of complement-fixing antibodies against the homologous streptococci was followed.

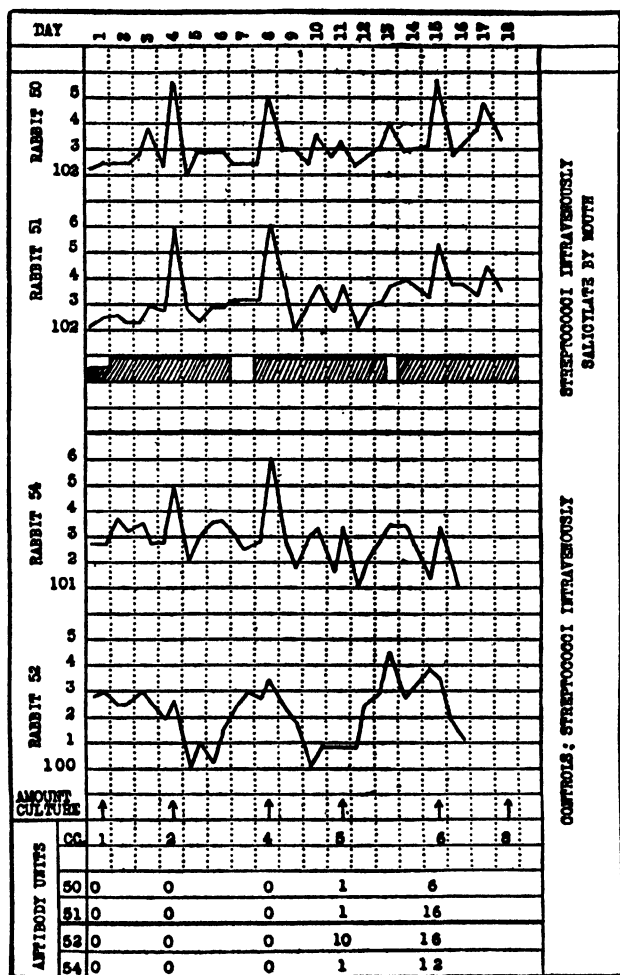
The antigens used in the tests were prepared by dissolving dried bacteria in antiformin and titrating this solution back to neutral according to the method described by Kinsella and Swift (3). The reactions were carried out in the usual manner, with a period of 1 hour for incubation of antigen, complement, and antibody; after which sensitized sheep red blood cells were added and the tubes returned to the water bath at 37°C. for 2 hours. Immediate readings were then made. The antigens were used in one-half the anticomplementary dose, and both the complement and hemolytic amboceptor in double the hemolytic dose.

Experiment 1.—Four rabbits of practically equal weight were chosen and weighed daily for a week; their temperatures were taken morning and evening as a control of the subsequent period. The animals were divided into two groups of two each. All received intravenous injections of living cultures of *Streptococcus viridans* A49 twice a week in amounts shown in Text-fig. 1. Rabbits 50 and 51 received daily sodium salicylate, 0.16 gm. per kilo of body weight. All of the sera were tested for complement-fixing antibodies on the same day with the same complement and antigen. The general results of the experiment are shown in Text-fig. 1.

It is evident that prolonged administration of sodium salicylate by stomach tube in the dose used had no deleterious effect on the rabbits. A loss of weight in all of the animals can be attributed to other factors than salicylates, such as the repeated inoculation with streptococci, or snuffles. Two control rabbits receiving the same amount of sodium salicylate, but not inoculated with streptococci, showed no loss in weight and little variation in the temperature curve.

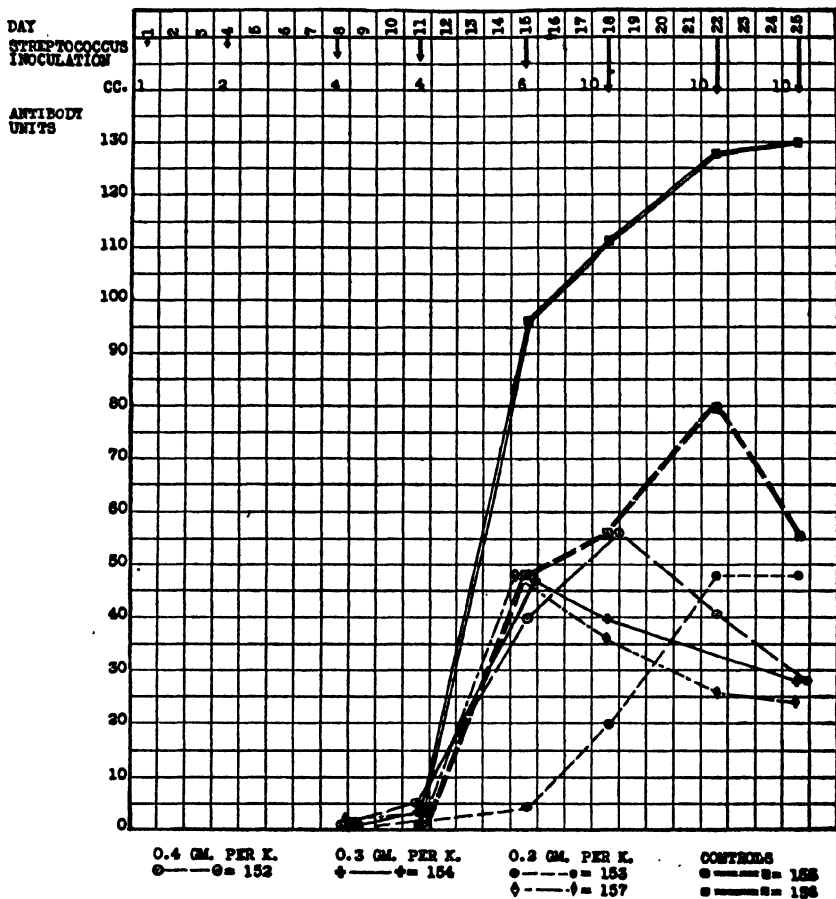
Apparently, the salicylates had little or no antipyretic effect, for the rabbits, whether receiving the drug or not, showed a similar fever within a few hours after intravenous inoculation of living streptococci. Rabbit 52 showed less effect following inoculation than did any of the others. The complement-fixing antibody is indicated in units. The first antibodies were detected in all of the rabbits on the 11th day following the first inoculation, when the two salicylated animals and one control showed equal titers; the other con-

trol had more antibodies. On the 15th day the combined non-salicylated controls yielded sera with stronger titer than did the salicylated animals. The experiment was terminated at this point because of the death of one of the controls.



TEXT-FIG. 1. Comparison of temperature curves and complement-binding antibody units in salicylated and control rabbits inoculated twice weekly with living *Streptococcus viridans* A49. The cross-hatching indicates periods of sodium salicylate administration.

Experiment 2.—Of six rabbits, two served as controls; the other four received sodium salicylate by stomach tube, two doses a day, as follows: No. 152, 0.4 gm. per kilo of body weight a day; No. 154, 0.3 gm.; Nos. 153 and 157, 0.2 gm. All of the animals were inoculated twice a week with increasing amounts of living



TEXT-FIG. 2. Comparison of complement-binding antibodies in rabbits receiving different amounts of sodium salicylate with controls; animals immunized twice weekly with living *Streptococcus viridans* A49.

Streptococcus viridans A49, as indicated in Text-fig. 2. Blood was obtained before each inoculation; the separated sera were stored until the termination of the experiment, and all were tested simultaneously against the homologous streptococcus for complement-fixing antibodies. The experiment was terminated after the 25th day; the results are shown in Text-fig. 2.

It is evident that there is no striking difference in the antibody content of the rabbits that received different amounts of sodium salicylate; the variation is no greater than might be seen in four different untreated rabbits. It is noteworthy that the two non-salicylated controls showed higher antibody curves than did any of the salicyl-treated animals.

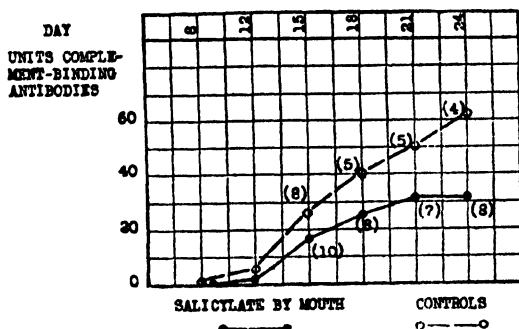
All of the animals were weighed, and all showed similar losses of from 100 to 200 gm.; therefore, in so far as this feature is concerned, neither deleterious nor beneficial effects could be attributed to the salicylates.

Experiment 3.—The same general plan was followed as in Experiment 1, with the following exception. The rabbits were immunized with a stock vaccine prepared by suspending in normal saline solution the centrifugate of a 24 hour broth culture of hemolytic *Streptococcus K*, and killing the bacteria by heating at 56°C. for $\frac{1}{2}$ hour. The animals were immunized by injecting intravenously increasing amounts of this vaccine. Temperature and weight curves of the rabbits were followed; only the temperature curves are reproduced, as the weights of three of the animals increased during the experiment, and that of the fourth was stationary. Blood was withdrawn before each inoculation; the sera were tested at two different periods, after the 18th and 32nd days, respectively. The number of complement-fixing units is recorded in Text-fig. 3.

It is evident that there was little if any difference in the antibody content of the two groups of animals; the difference is no greater than might be expected in such small groups. The fever normally following the intravenous injections of bacterial vaccines was uninfluenced by the administration of salicylates in the same manner as was the fever following the intravenous inoculation of living *Streptococcus viridans*.

A similar experiment was conducted in which the rabbits were inoculated with living cultures of hemolytic *Streptococcus K*. These rabbits died from generalized streptococcus infection before sufficient time had elapsed to obtain comparable results. Here, again, no distinct antipyretic effect was seen in the salicyl-treated animals as compared with untreated controls. All four of the rabbits inoculated with living hemolytic streptococci had multiple purulent arthritis from which pure cultures of hemolytic streptococci were recovered. It is therefore evident that the salicylates had no influence in preventing the development of arthritis.

A summary of all of the experiments showing the influence of salicylates upon the formation of complement-fixing antibodies is given in Text-fig. 4. Here the averages derived from a larger group of animals than was used in any individual experiment indicates that salicylates in general had a depressing influence upon the formation of this type of immune bodies.



TEXT-FIG. 4. Comparison of average complement-binding antibody formation in the foregoing series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

Influence of Salicylates on the Formation of Agglutinins.

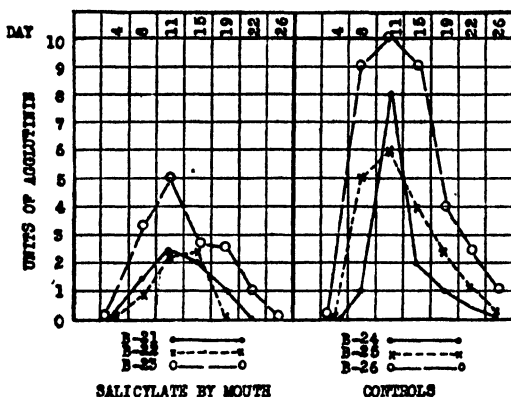
In the next four experiments the rate of agglutinin formation was determined.

In all experiments the sera were tested against killed cultures of the homologous bacteria prepared as follows: A 24 hour broth culture was heated at 56°C. $\frac{1}{2}$ hour, and standardized by Gates' (4) method so that the density of the suspensions was similar. The reaction of the suspensions was brought to a pH 7.3 or 7.4 to avoid acid agglutination. In order to insure that the suspending medium was the same in all of the tubes the sera were diluted with broth, instead of salt solution. The tubes were incubated at 56°C. for 2 hours, removed from the incubator, and allowed to cool for 15 minutes; then the strength of reaction was recorded.

Experiment 4.—Six brown rabbits weighing from 1,400 to 1,600 gm. were immunized with a pneumococcus of Type I by giving daily intravenous injections of vaccines prepared by suspending the centrifugate of 24 hour broth cultures in normal saline solution and heating at 56°C. for $\frac{1}{2}$ hour. An amount of vaccine representing 2 cc. of culture was given to each rabbit daily for 6 days. Three of the rabbits (Nos. B-21, B-22, and B-23) received sodium salicylate daily for

14 days; the dose was 0.16 gm. per kilo of body weight. The other three rabbits served as controls. The sera of all of the animals were tested for agglutinin content against the same suspension of homologous pneumococci. The results are given in Text-fig. 5.

In this experiment the amount of immunizing antigen was small and the period of immunization was short in comparison with the previous experiments. It is difficult to obtain in rabbits an agglutinating serum of high titer against pneumococci. In this series, therefore, the curves are low; the salicylated rabbits, nevertheless, had uniformly less antibodies than did the controls.



TEXT-FIG. 5. Comparison of agglutinin formation in salicylated and control rabbits immunized with six daily injections of pneumococcus vaccine.

Experiment 5.—Ten brown rabbits weighing between 1,700 and 1,800 gm. each were divided into two lots of five. One lot was given sodium salicylate, 0.16 gm. per kilo of body weight, daily for 20 days. On the 2nd day all of the animals received intravenously the centrifugalized sediment of 5 cc. of broth culture of *Streptococcus viridans* 38D; on the 6th day a similar amount of the same organism; and on the 14th day 10 cc. Blood was obtained from the animals before the beginning of treatment and on the 6th, 10th, 14th, 17th, and 21st days. Three of the salicylate-treated animals and one of the control group died during the course of the experiment; all of the surviving animals were killed and examined for lesions of the joints and viscera on the 20th or 21st day. At the termination of the experiment all of the sera were titrated for agglutinins. The final interpolated titers of the various sera are given in Table I.

TABLE I.

Comparison of Agglutinin Formation in Salicylated and Control Rabbits Inoculated with Living Streptococcus viridans 38D.

Date.	Day.	Inoculation.	Salicylated rabbits.							Controls.						
			A-52	A-54	A-56	A-58	A-60	A-62	Aver- age.	A-53	A-55	A-57	A-59	A-61	A-65	Aver- age.
1921																
Apr. 2	2	5 cc. of Streptococcus 38D.	0	0	0	0	0	0		0	0	0	0	0	0	
" 7																
" 11	6		10	8	10	20	18	18	14	27	8	18	16	20	17	18
" 11	6	5 cc. of Streptococcus 38D.														
" 15	10		18	10	30	120	96	64	56	76	20	80	96	70	76	69
" 18	10	10 cc. of Streptococcus 38D.														
" 19	14		224	176	256	480	320	Dead.	291	480	80	480	1,120	224	880	544
" 22	17		510	304	Dead.	512	560	"	471	640	152	512	1,200	Dead.	1,280	757
" 26	21		510	640	"	1,200	Dead.	"	783	350	1,040	880	2,240	"	2,400	1,382

Although the usual variation in the agglutination titer in the serum of different animals is seen in both groups; most of the rabbits in the control group consistently yielded stronger agglutinating serum than did the salicyl-treated rabbits; the average curve of the control group was uniformly higher than that of the salicyl group, and on the 14th, 17th, and 21st days it was nearly twice as high.

In another experiment carried out in a manner similar to No. 5, part of the salicylated rabbits succumbed to intravenous inoculations of *Streptococcus* A49; on the 8th day the three surviving rabbits in this group gave an average agglutinin content of 46 units; and six controls gave an average content of 57 units. On the 16th day only a single salicylated animal was living, while five controls still survived; at this time both groups had an average agglutinin content of 900. Because of the difference in the number of animals in the two groups this experiment is not given in detail. It merely shows that a single salicylated animal may have as high an antibody curve as some untreated controls; and emphasizes the desirability of having more than one animal in each group.

In still another experiment in which only two salicylated rabbits and four controls lived until the 6th day, the average agglutinin curve on this day was 735 for the first and 450 for the second group. These animals were immunized with a single intravenous injection of 35 cc. of *Streptococcus viridans* A135. The difference in the number of animals in the two groups also decreases the value of the results in this experiment. It is possible that the large amount of immunizing antigen may have been a factor in eliminating the difference in the two groups of animals.

These two experiments were the only ones in which the animals receiving salicylates yielded serum with as high or higher average antibody content than that of control animals.

Experiment 6.—This experiment was designed to determine whether salicylate-treated rabbits and non-salicylated controls, all previously inoculated into the knee joints with killed cultures of green streptococci, would show any difference in the agglutinating power of their sera after intravenous inoculation with living streptococci.

Each of six brown rabbits weighing between 1,600 and 1,900 gm. was inoculated into the right knee with the sediment of 0.5 cc. of broth culture of *Streptococcus viridans* 38D killed by heating for $\frac{1}{2}$ hour at 56°C. and into the left knee with 0.5 cc. of *Streptococcus viridans* A49 similarly treated. The knees were measured and otherwise observed until they had reached their original dimensions or were stationary in size. Serum was separated from blood obtained on the 3rd, 20th, and 28th days following intraarticular inoculation. The animals were then divided into two groups of three each, and the members of one group given

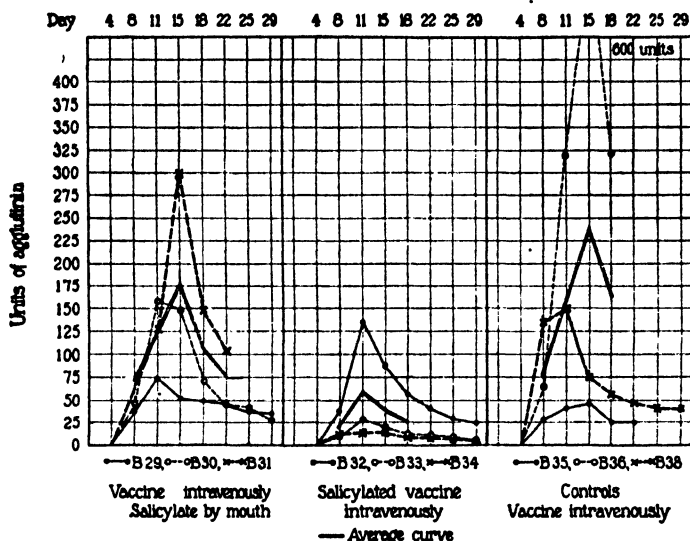
sodium salicylate daily in doses of 0.2 gm. per kilo of body weight; this treatment was started on the 27th day after the intraarticular inoculation. The next day each of the animals received an intravenous injection of the sediment of living streptococci from 9.3 cc. of an 18 hour culture of *Streptococcus* 38D. Serum was obtained on the 3rd and 9th days following this intravenous inoculation, and on the 9th day all of the animals were autopsied to determine the extent of articular and visceral lesions.

In the immunity studies several points may be noted: (1) the possibility of detecting agglutinins in the blood following the intraarticular inoculation of small amounts of two different strains of killed streptococci; (2) the effect of subsequent intravenous inoculation of one of the two strains—whether it stimulates antibodies to one or both; and (3) the difference in the salicylate-treated and control groups. The results are summarized in Table II.

Although the antibody curve was not followed at frequent intervals after the intraarticular injection of killed streptococci, it is evident that a single injection of Strain A49 stimulated the formation of agglutinins much more than did a simultaneous injection of Strain 38D. It is impossible to state whether the agglutinins detected on the 28th day were stimulated by the intraarticular injection or by the intravenous inoculation because the blood was withdrawn 8 hours after the intravenous inoculation. The increase in agglutinins for Strain A49 on the 9th day after the intravenous injection of Strain 38D must have been due to the stimulating effect of the latter strain in animals already slightly immune to No. A49. The rise in antibody curve at this time was noted for both strains. The two strains are distinct antigenically; the serum of many animals immunized alone with Strain 38D has never agglutinated No. A49, and *vice versa*. The increase in antibodies for No. A49 on the last day must, therefore, have been a concomitant phenomenon, and did not seem to have been influenced by salicylates. The lower agglutinin content for *Streptococcus* 38D in the salicylated animals is similar to that observed in other experiments.

Experiment 7.—The object of this experiment was to determine in addition whether streptococci treated *in vitro* with salicylates were less active antigenically than untreated streptococci. Three groups of animals were chosen. Group 1, consisting of Rabbits B-29, B-30, and B-31, received sodium salicylate by stomach

tube, 0.2 gm. per kilo of body weight, and daily injections of vaccine prepared from broth cultures of *Streptococcus viridans* 38D. The vaccine was given for 5 days. Group 2, consisting of Rabbits B-32, B-33, and B-34, received daily intravenous injections of salicylated vaccine. This vaccine was prepared as follows: An aliquot portion of the vaccine used in immunizing Group 1 was mixed with an amount of sodium salicylate solution that would have been used had the rabbit received the drug by stomach tube. The vaccine and salicylate were incubated at 37°C. for 4 hours, then centrifugalized, and the centrifugate was taken up in saline solution and injected intravenously. This vaccine was given for 5 days. Group 3, the control group, consisting of Rabbits B-35, B-36, and B-38, received



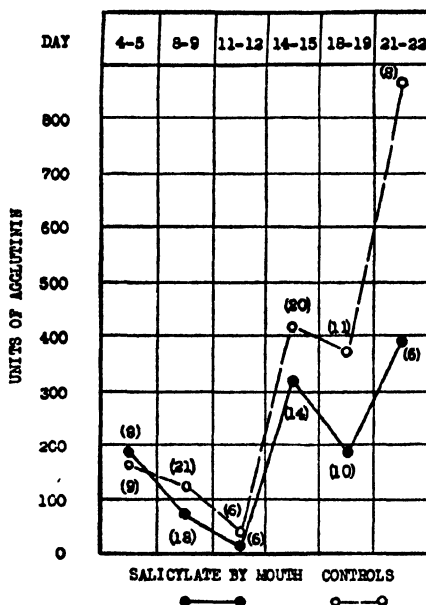
TEXT-FIG. 6. Comparison of agglutinin formation in salicylated and control rabbits immunized with vaccines of *Streptococcus viridans* 38D, and rabbits immunized with salicylated vaccines.

daily injections of untreated vaccine. All of the rabbits received each day vaccines in an amount equal to 2 cc. of broth culture per kilo of body weight. The rabbits were bled before the experiment and on other days indicated in Text-fig. 6. All of the sera were tested for agglutinin content on the same day. The results are shown in Text-fig. 6.

Here, as in previous experiments, the control group had a higher agglutinin content in their sera than did the rabbits treated with sodium salicylate. The second group, namely those that received salicylated vaccine, had the lowest antibody content. In this experi-

ment it seemed, therefore, that the action of the salicylate might be directly upon the antigen, causing it to be a less active stimulator of antibody production in the animal body.

It was thought advisable to determine whether streptococci treated with salicylate might act differently towards an agglutinating anti-



TEXT-FIG. 7. Comparison of average agglutinin formation in the complete series of salicylated and control rabbits. The number of animals tested each day is indicated in parenthesis.

serum than untreated streptococci. The following experiment was therefore performed.

A 24 hour broth culture of *Streptococcus* 38D was killed by heating $\frac{1}{2}$ hour to 56°C. The reaction of the medium at this time was pH 7.4. The culture was divided into two parts: X was kept in the original state; Y was treated as follows: It was centrifugalized and the supernatant serum broth retained. The separated bacterial sediment was mixed with 400 cc. of 5 per cent sodium salicylate and incubated for 4 hours at 37°C. It was then centrifugalized to clearness, the supernatant sodium salicylate solution discarded, and the bacterial sediment added to the original broth. The resuspension of the cocci in the medium in which they were grown insures that Suspensions X and Y were the same except that Suspension Y contained salicylated streptococci. These two suspensions were

then tested against the sera of Rabbits B-29, B-31, B-32, B-33, and B-34. With each serum the agglutination reached the same point in both suspensions; the differences in the agglutinating power of the serum of the rabbits of Groups 1 and 2 cannot, therefore, be attributed to the possibility that Group 2 rabbits were immunized with less agglutinable streptococci.

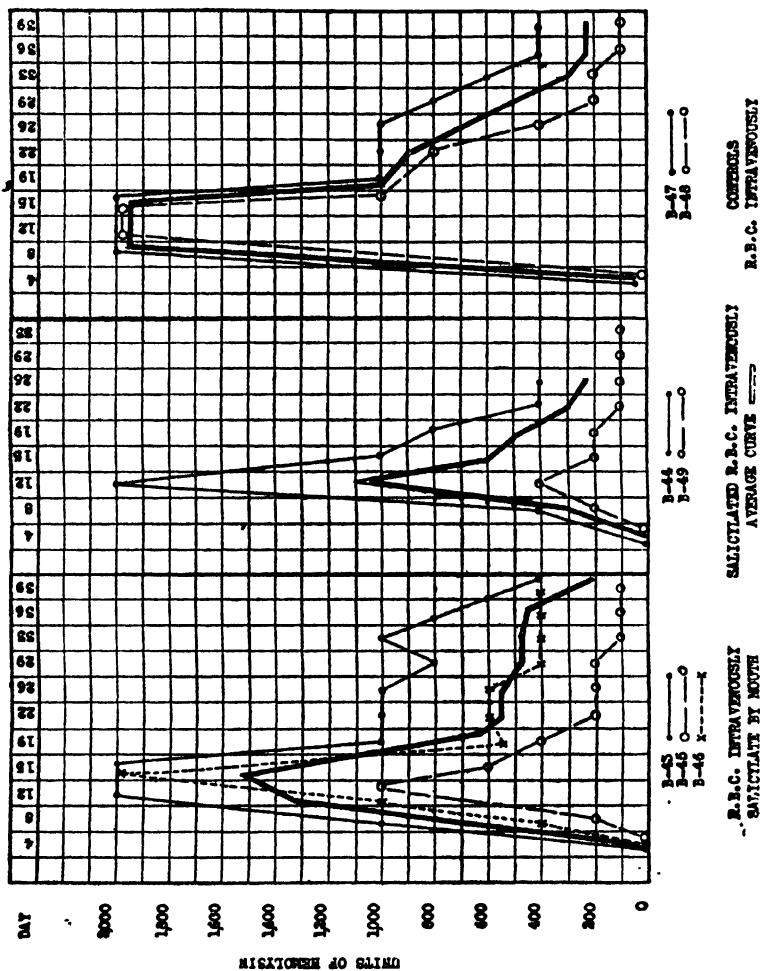
The average agglutinin content of the serum of all of the rabbits used in the foregoing experiments is shown in Text-fig. 7. The irregular course of the curves is due to the difference in the number of animals tested at different periods. At each period, however, the number of controls is equal to or greater than the number of salicylated animals. This summary shows that the average agglutinin formation in the entire group of salicylated animals was lower than that of the controls, and is in accord with the findings of the animals tested for complement-binding antibodies.

Influence of Salicylates on the Formation of Hemolysin.

In the remaining experiments the antibody tested was an hemolysin against sheep red blood cells.

All of the sera were inactivated by heating at 56°C. for $\frac{1}{2}$ hour. The diluted serum was then mixed with guinea pig serum so diluted that 0.5 cc. equaled one unit of complement previously tested with a constant standard hemolytic serum. To the rabbit serum and guinea pig complement was added 0.5 cc. of a 5 per cent suspension of sheep red blood cells. The mixtures were incubated in the water bath at 37°C. for 2 hours and read immediately. The dilution at which hemolysis was complete was considered as containing the charted unit of antibody. Because of the result obtained in Experiment 7 the experiments with hemolysins were all carried out with three groups of animals: Group 1, in which the animals received washed sheep red blood cells intravenously and sodium salicylate by stomach tube; Group 2, in which the animals received intravenous injections of salicylated sheep red blood cells, prepared by incubating together washed erythrocytes and a 2.5 per cent sodium salicylate solution, then centrifugalizing the mixture and retaining the cells alone for inoculation; Group 3, in which the animals received simply intravenous injections of washed sheep red blood cells.

Experiment 8.—Seven rabbits about 6 weeks old, all brown, of the same litter, were chosen for this experiment. Inactivated serum obtained from a preliminary bleeding was tested for hemolysin content against sheep red blood cells. The rabbits were then divided into three groups according to their weight and the native hemolysin content of their serum. This precaution was taken because certain investigators claim that the amount of antibody formation following



TEXT-FIG. 8. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes. Salicylate and erythrocytes given daily for 3 days, 1 day intermission, then for 3 more days.

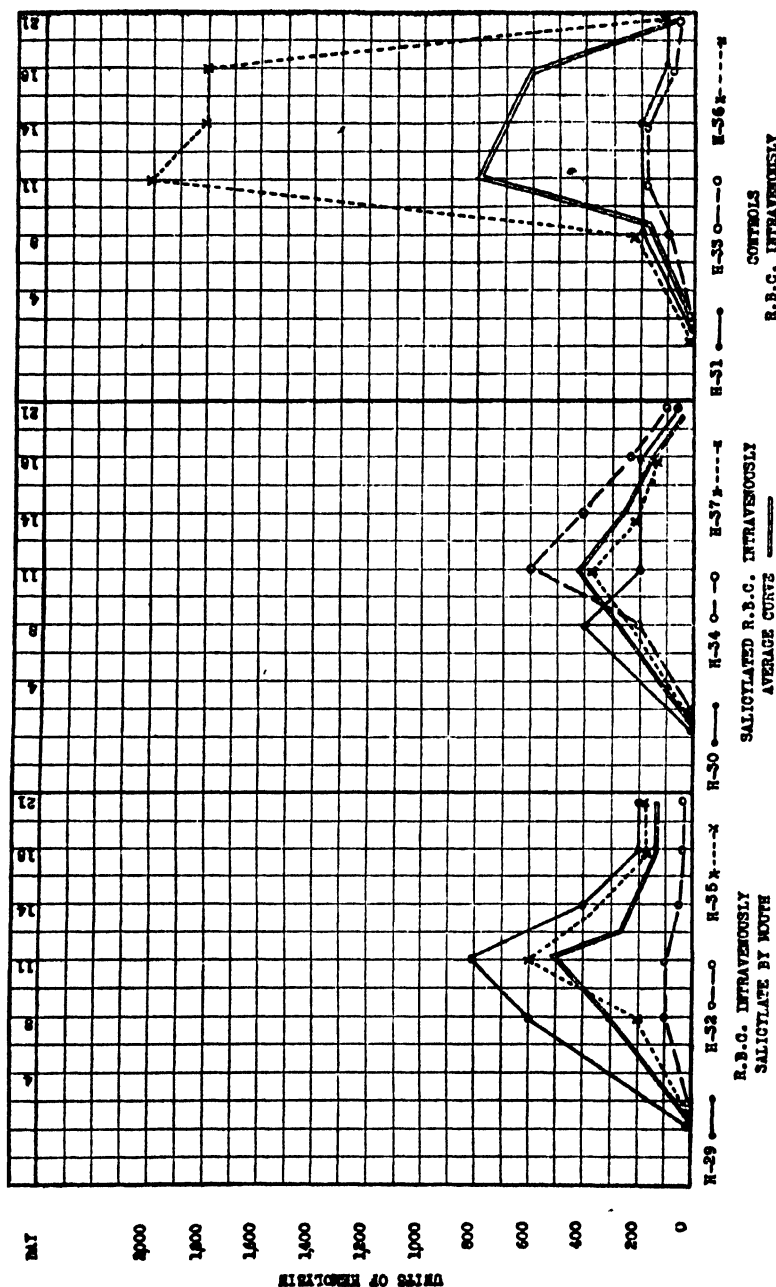
immunization is more or less in direct proportion to the amount of antibody the serum of the animal contained before immunization. Group 1, consisting of Rabbits B-43, B-45, and B-46, received injections of sheep cells daily for 3 days followed by an interval of 1 day, and then for 3 more days, and at the same time sodium salicylate by stomach tube—a total daily amount of 0.16 gm. per kilo of body weight per day. Group 2, consisting of Rabbits B-44 and B-49, received intravenous injections on the same days as did rabbits of Group 1. They received sheep red blood cells that had been incubated for 3 hours with sodium salicylate, the supernatant salicylate solution discarded, and the salicylated cells resuspended in saline solution. Group 3, controls, consisting of Rabbits B-47 and B-48, was injected with washed sheep blood cells. The antibody curves of these rabbits are shown in Text-fig. 8.

In all three groups some of the rabbits showed hemolysin titers as high as 1,000 to 2,000. Comparing the average curve for each group, however, the highest and most prolonged antibody content was found in Group 3, the next in Group 1, and the lowest in Group 2, the group immunized with salicylated cells.

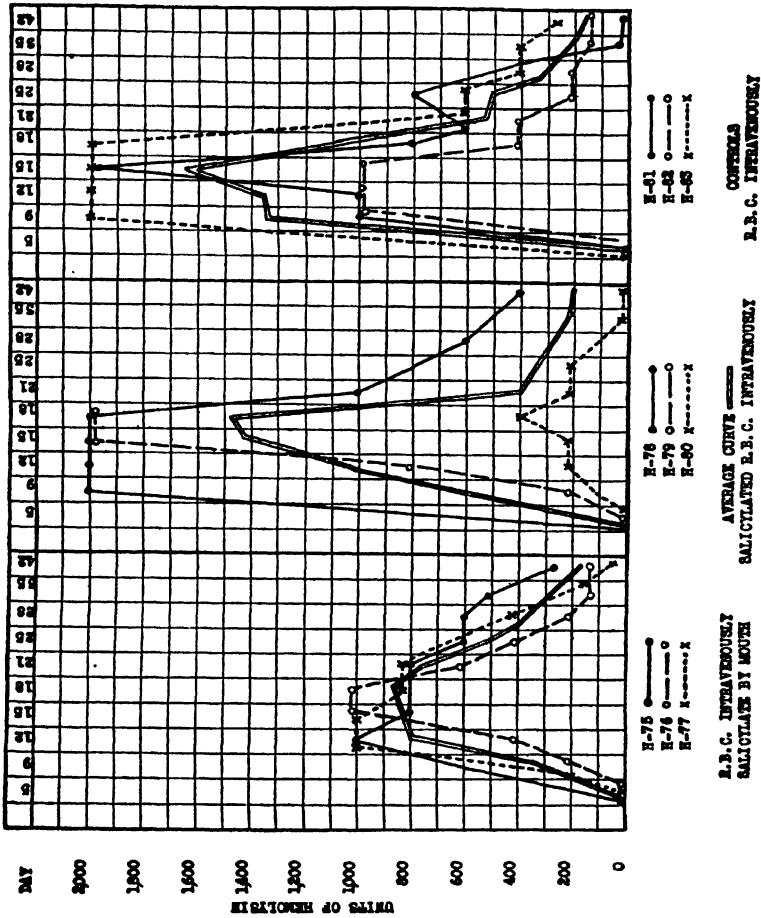
Experiment 9.—This experiment was carried out in essentially the same manner as Experiment 8, with the following exceptions. Three groups of larger rabbits, consisting of three animals each, were used; injections of erythrocytes were given only 3 days; and the sodium salicylate by mouth was given for a similar period. The influence of the salicylate was, therefore, correspondingly less than in animals receiving the drug over a longer period. The results are shown in Text-fig. 9.

In this experiment the differences between the various groups were less marked than in previous experiments although the average curve in the control group (Group 3) was higher than in Groups 1 and 2. This high average is due to the fact that the serum of Rabbit H-36 had a much higher antibody content than that of any of the others. The other two rabbits in this group had a lower antibody content than any of the rabbits in the other two groups. As in Experiment 8 the animals receiving salicylate by stomach tube had a higher average curve than did those receiving salicylated red blood cells intravenously.

Experiment 10.—A comparison of the results in Experiments 8 and 9 seems to indicate that a long or short period of administration of the salicylates might be a factor in determining the degree of antibody formation. Experiment 10 was made to test the influence of more prolonged administration of the drug. The rabbits used in this experiment were all brown, full grown, and of approximately

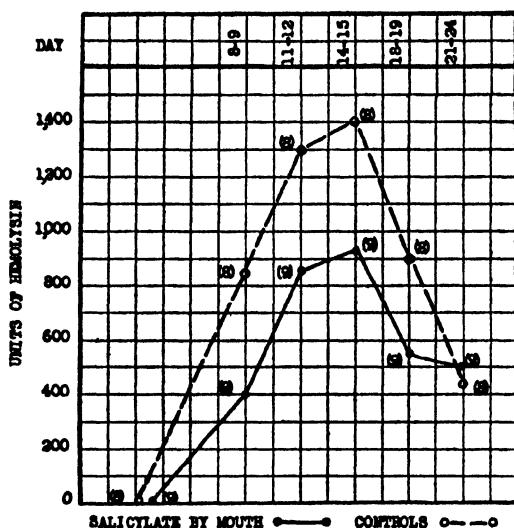


TEXT-FIG. 9. Comparison of hemolysis formation in salicylated and control rabbits immunized with sheep erythrocytes, and rabbits immunized with salicylated erythrocytes. Salicylate and erythrocytes given daily for 3 days.



TEXT-FIG. 10. Comparison of hemolysin formation in salicylated and control rabbits immunized with sheep erythrocytes and rabbits immunized with salicylated erythrocytes. Salicylate given for 12 days; erythrocytes injected on the 1st, 5th, and 9th days.

the same size. Three intravenous injections of 25 per cent sheep red blood cells, 4 cc. per kilo of body weight, were given to each rabbit on the 1st, 5th, and 9th days, respectively. In Groups 1 and 3 the cells were not salicylated; in Group 2 the cells were salicylated by incubating them with 2.5 per cent sodium salicylate solution for 15 hours, the suspension centrifugalized, the supernatant solution discarded, and the cells resuspended in saline solution. Group 1 animals received sodium salicylate in a total daily amount of 0.2 gm. per kilo of body weight. The animals received three doses before the first immunizing cells were given and were treated 4 days after the last intravenous injection of erythrocytes. The results are shown in Text-fig. 10.



TEXT-FIG. 11. Comparison of average hemolysin formation in the salicylated and control rabbits used in Experiments 8 to 10. The number of rabbits tested each day is indicated in parenthesis.

Here, as in previous experiments, the control group had a higher total as well as higher average antibody content than did the other two groups. Group 1, in which the rabbits received salicylate for 12 days, showed the lowest antibody content. Group 2, treated with salicylated cells, showed almost as great an antibody content as did Group 3. The discrepancy between the animals in Group 2 in this experiment and in the three previous experiments cannot be explained.

If the average hemolysin content of the sera of all of the animals used in Experiments 8 to 10 are plotted (Text-fig. 11), it is seen that

the control animals not receiving salicylate yielded sera with consistently higher titer than did animals receiving salicylate by mouth. This is in complete accord with the results obtained for agglutinating and complement-binding antibodies.

DISCUSSION.

Aside from the two incomplete experiments in which the number of animals in the salicylated group was small, the controls all had a higher antibody content than did animals similarly immunized but treated with sodium salicylate. The dose of salicylate given the rabbits each day was comparable with the largest amount that may be safely administered to patients for short periods, and much larger than can be tolerated over a long period by patients with rheumatic fever. While some rabbits receiving 0.4 gm. per kilo each day seemed to suffer no deleterious effects, others receiving this amount of the drug succumbed more quickly to intravenous inoculation of living streptococci than did unsalicylated controls.

Fantus, Simmonds, and Moore (5) showed that sodium salicylate, when used in a dose which they state was comparatively harmless to animal controls, is decidedly detrimental and liable to be fatal to rabbits infected with hemolytic streptococci. In their experiments from 0.9 to 1 gm. of sodium salicylate and 1.2 gm. of acetylsalicylic acid were given by mouth each day; in another series 0.3 gm. of sodium salicylate was given hypodermically. These are much larger doses than may be given with safety to patients. Our experiments indicate that 0.2 gm. is as large a daily dose as can be safely administered to rabbits over long periods.

Rabbits receiving the drug daily by stomach tube at first refused to take much food, but after 3 or 4 days they again resumed their normal feeding habits. Malnutrition in this group of animals cannot, therefore, be considered as the cause of a lowered antibody formation.

It is possible that animals with less febrile response following the intravenous injection of antigens might show a correspondingly lower antibody formation. As the salicylates have a marked antipyretic effect in patients this argument might be used theoretically to explain our results. In the first and third experiments it was shown, however, that salicylates in the doses employed did not have an anti-

pyretic effect in inoculated rabbits. A hypothetical correlation between fever and antibody formation must, therefore, be abandoned in explaining the results.

Only a few drugs or chemicals have been tested to determine their action upon the formation of immune bodies.

With the introduction of salvarsan several workers (6, 7) claimed that one of the marked actions of arsenicals in general was to increase antibodies. Others (8, 9) have claimed that this effect was less marked and less constant than found by the earlier experimenters. Toyama and Kolmer (9) state that large doses of both salvarsan and mercury bichloride appear to depress hemolysin and agglutinin production in immunized rabbits; while smaller doses of both drugs tend to increase the production of hemagglutinins, but not of hemolysins.

Müller (10) found that animals treated with aleuronat or cinnamic acid while undergoing immunization yielded more antibodies than did controls. He thought that because these two substances stimulated leucocytosis the two effects were to be attributed to a common action. Similarly the depression in immune bodies in animals under the influence of benzene led Hektoen (11) to believe that substances exerting a destructive action on the blood-forming organs had a depressing effect on the tissues from which immune bodies arise. But his later studies concerning the effect of toluene (12) and thorium (13) on immune body production indicate that variations in the antibody content of the blood serum and leucocytosis or leucopenia need not necessarily go hand in hand.

Abbott and Bergey (14), Müller (10), and Friedberger (15) have all demonstrated conclusively that alcohol in repeated doses has a very depressing influence on the capacity of immunized animals to form antibodies. Melnikowa and Wersilowa (16), and Müller (10) have shown that phlorhizin has a similar effect. Hektoen and Corper (17) have shown that mustard gas (dichloroethylsulfide) depresses immune body formation.

The foregoing review of previous experiments fails to furnish any explanation that can be brought to bear upon our studies.

Cook (18) has shown that the rate of antibody formation is dependent upon the rate of absorption of the antigen. She reviews and makes use of the work of Loeb, Lillie, and Osterhout, all of whom have demonstrated that the permeability of cells is increased by increasing the sodium ions and decreased by increasing the calcium ions. In her experiments, rabbits treated with sodium citrate formed antibodies more intensely than did controls, and those treated with calcium chloride less intensely than controls. In all of our experiments the rabbits receiving salicylates were at the same time subject to an

increased amount of sodium from the sodium salicylate. From Cook's experiments one would expect an increased antibody output if the sodium ion is the important element in the compound. Whether the salicyl ion has an antagonistic effect by decreasing the absorption of antigen is a question that must be submitted to experimental study. Reasoning simply by similarity of effect it seems possible that the decreased antibody production in rabbits treated with sodium salicylate may be due to the decreased power of these animals to absorb antigen.

It must be emphasized in this connection that there is nothing in our experimental results to indicate that the salicylates are deleterious in infection simply because they decrease immune body formation. In none of the experiments was this decrease extremely marked, and all of the salicylated animals produced immune bodies in fair amounts. It is probable that immune bodies in the circulating fluids are not the only factors concerned in the process of recovery. The same mechanism that makes an infectious agent less antigenic might at the same time make it less pathogenic. It is possible that this effect may be more marked in rheumatic fever than in other infections; hence the more striking effect of the drug seen in this disease.

CONCLUSION.

1. Rabbits treated with sodium salicylate in daily doses of from 0.16 to 0.2 gm. per kilo of body weight and at the same time immunized with intravenous injections of *Streptococcus viridans*, both living and in the form of vaccines, and also with washed sheep red blood cells, showed diminished complement-fixing antibodies, agglutinins, and hemolysins when compared with controls similarly immunized.

2. If the antigens were treated with sodium salicylate *in vitro* and subsequently injected intravenously into rabbits, the animals usually showed lower antibody curves than did rabbits that received the untreated antigen intravenously and sodium salicylate by stomach tube.

3. The beneficial effect of sodium salicylate in rheumatic fever patients probably cannot be attributed to an increased production

of circulating immune bodies against the infectious agent. This is, however, no contraindication to the administration of salicylates to patients suffering from infectious diseases.

BIBLIOGRAPHY.

1. Barbour, H. G., *Arch. Int. Med.*, 1919, xxiv, 624. Barbour, H. G., and Devenie, M. M., *Arch. Int. Med.*, 1919, xxiv, 617.
2. Jacoby, M., and Schütze, A., *Biochem. Z.*, 1908, ix, 527.
3. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1917, xxv, 877.
4. Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
5. Fantus, B., Simmonds, W. E., and Moore, J. J., *Arch. Int. Med.*, 1917, xix, 529.
6. Friedberger, E., and Masuda, N., *Therap. Monatsh.*, 1911, xxv, 288.
7. Boehncke, K. E., *Z. Chemotherapie., Orig.*, 1912-13, i, 136.
8. Reiter, H., *Z. Immunitätsforsch., Orig.*, 1912, xv, 116.
9. Toyama, I., and Kolmer, J. A., *J. Immunol.*, 1918, iii, 301.
10. Müller, P. T., *Arch. Hyg.*, 1904, li, 365.
11. Hektoen, L., *J. Infect. Dis.*, 1916, xix, 69.
12. Hektoen, L., *J. Infect. Dis.*, 1916, xix, 737.
13. Hektoen, L., and Corper, H. J., *J. Infect. Dis.*, 1920, xxvi, 330.
14. Abbott, A. C., and Bergey, D. H., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxii, 260.
15. Friedberger, E., *Berl. klin. Woch.*, 1904, xli, 242.
16. Melnikowa, F. J., and Wersilowa, M. A., *Centr. Bakt., 1te Abt., Orig.*, 1912, lxvi, 525.
17. Hektoen, L., and Corper, H. J., *J. Infect. Dis.*, 1921, xxviii, 279.
18. Cook, M. W., *J. Immunol.*, 1920, v, 39.

THE ARTHRITIS OF SERUM SICKNESS.

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Most patients receiving considerable amounts of therapeutic serum develop serum sickness. Formerly this was most frequently observed following the administration of diphtheria antitoxin, but recently the introduction of larger doses of horse serum in the treatment of pneumonia and cerebrospinal meningitis has furnished more examples of this condition. Clinically, it is manifested during the second and third weeks following the serum treatment by fever, lymph node enlargement, urticaria and short, transitory leukopenia followed by leukocytosis. Another less frequent but very distressing symptom is joint pain and tenderness, the exact nature of which is but little understood. Up to the present time this manifestation has taken its name from the subjective symptoms.

Pirquet and Schick¹ speak of it as *Gelenkschmerzen*. During the treatment of a large series of patients with antistreptococcic and antidiphtheritic serums, they did not encounter a single instance in which there was clinically demonstrable exudation into the joints. Hartung² found joint manifestations in ten among 375 serum treated patients (ten of sixty-eight cases of serum sickness). In eight of these ten cases, one or more joints showed swelling. The joints most often involved were those of the hands; in two instances, the right knee was swollen, the patella floated, and the skin was stretched and reddened. Goodall,³ in describing the "complications" of serum sickness, states that "the most frequent of these is one which usually goes by the name of 'joint pains,' but which is, I have no doubt, an arthritis." In certain of his cases, swelling of the joints occurred, but this was extremely rare; in the majority there was only pain with stiffness.

¹ Pirquet, C., and Schick, B.: Die Serumkrankheit, 1905.

² Hartung, C.: Jahrb. f. Kinderh. 42: 72, 1896.

³ Goodall, E. W.: Lancet 1: 323 (March 2) 1918.

In only one of 376 patients having joint involvement did a relapse of arthralgia occur. Longcope⁴ describes the condition under the term arthralgia. He says: "In comparison with the severity of the arthralgia, there is little to be observed. The joints are somewhat tender to the touch, but there is not, except in rare instances, swelling or reddening, and the joints are not hot to the touch. In only one instance have I seen a slightly swollen and red wrist." More or less joint symptoms were noted in about 20 per cent of the cases of serum sickness observed by Weaver.⁵ He found the pains to be spontaneous, and increased by motion and sometimes by touch. The severity of the pains was in marked contrast to the limited objective findings, and the pain disappeared in from one to three days, leaving the joints without permanent alteration. In his experience, swelling of the affected joints was unusual. Von Ritterstein⁶ observed one case with swelling of the joints. Poynton and Paine⁷ state that "antitoxic sera free from bacteria may sometimes produce effusion into the joints." Bécclère, Chambon and Ménard⁸ observed urticaria and symptoms of joint lesions in calves injected with horse serum. They state that microscopic examination of sections of the skin and joints revealed no evidence of inflammation.

Weaver⁵ found that with doses of horse serum under 100 c.c. the frequency and intensity of serum disease was more or less in direct proportion to the amount introduced. Longcope and Rackemann,⁹ and Mackenzie and Leake¹⁰ have demonstrated that individuals receiving large amounts of serum fall into different classes according to their ability to react to the injection of the foreign protein. The majority produce antibodies readily and have severe symptoms; a smaller group produces no circulating antibodies and remains free from symptoms; the third group is intermediate between the other two.

⁴ Longcope, W. T.: Nelson Loose Leaf System of Medicine 2: 632.

⁵ Weaver, G. H.: Serum Disease, Arch. Int. Med. 3: 485 (June) 1909.

⁶ Von Ritterstein: Jahrb. f. Kinderh. 4: 542, 1902.

⁷ Poynton and Paine: Researches on Rheumatism, 1913, p. 230.

⁸ Bécclère, Chambon and Ménard: Ann. de l'Inst. Pasteur 10: 567, 1896.

⁹ Longcope, W. T., and Rackemann, F. M.: J. Exper. Med. 27: 341 (March) 1918.

¹⁰ Mackenzie, G. M., and Leake, W. H.: J. Exper. Med. 33: 601 (May) 1921.

In this hospital the occurrence of serum disease in patients treated intravenously with large amounts of Type I antipneumococcus serum offered an opportunity to investigate the character of this joint involvement and to compare the joint symptoms with the arthritis found in patients with rheumatic fever.

Ten cases of serum sickness have occurred in the last six months, six of which have shown mild or severe arthritic symptoms. One patient had pain only in the right shoulder; a second had pain in both shoulders and knees. Aspiration of the knees did not demonstrate an exudate. The remaining four had more severe joint symptoms extending over a longer period of time. In each, an exudate was demonstrated in one or more joints. In two of these, the exudates were evident both clinically and on aspiration; in the other two, no clinical signs of increased fluid could be determined, but aspiration yielded exudates. These last four cases were studied:

REPORT OF CASES.

CASE 1.—J. H., a man, aged 29, was admitted, Jan. 10, 1922, on the first day of pneumonia. Physical signs indicated involvement of the left lower lobe. Blood culture yielded pneumococci, Type I. During the second day, 300 c.c. of antipneumococcus serum (Type I, New York State Board of Health, Lot 187 A) was given intravenously. On each of the three succeeding days, 100 c.c. of this serum was similarly administered. The patient's condition improved. By the sixth day, signs of resolution had appeared, and the patient's general condition was good. Serum disease began on the eighth day with a general urticaria, which persisted six days. There was no general glandular enlargement. Joint symptoms appeared on the third day of the serum disease, and continued for fourteen days. These were severe, as can be seen in Chart 1, especially in the left wrist and the left metacarpophalangeal knee and ankle joints. The right knee joint was aspirated on the ninth day of serum sickness, and 13 c.c. of rather turbid, opalescent fluid removed. On the ninth, tenth and twelfth days the left knee was aspirated and exudate recovered each time. Early, this exudate was turbid and opalescent, but later it was greenish-yellow, and only faintly turbid. Precipitin tests made by Dr. W. T. Longcope for the presence of horse serum in the joint exudate revealed, on the ninth day, 1:1,000; tenth day, 1:100, and twelfth day, 1:10. On the tenth day of the arthritis the patient was having so much pain that large doses of sodium salicylate were administered; this was followed by marked improvement. Salicylates were discontinued abruptly without the recurrence of joint symptoms. There was no evidence of cardiac complications. The patient has continued well since discharge from the hospital. He gave no previous history of rheumatic fever or arthritis.

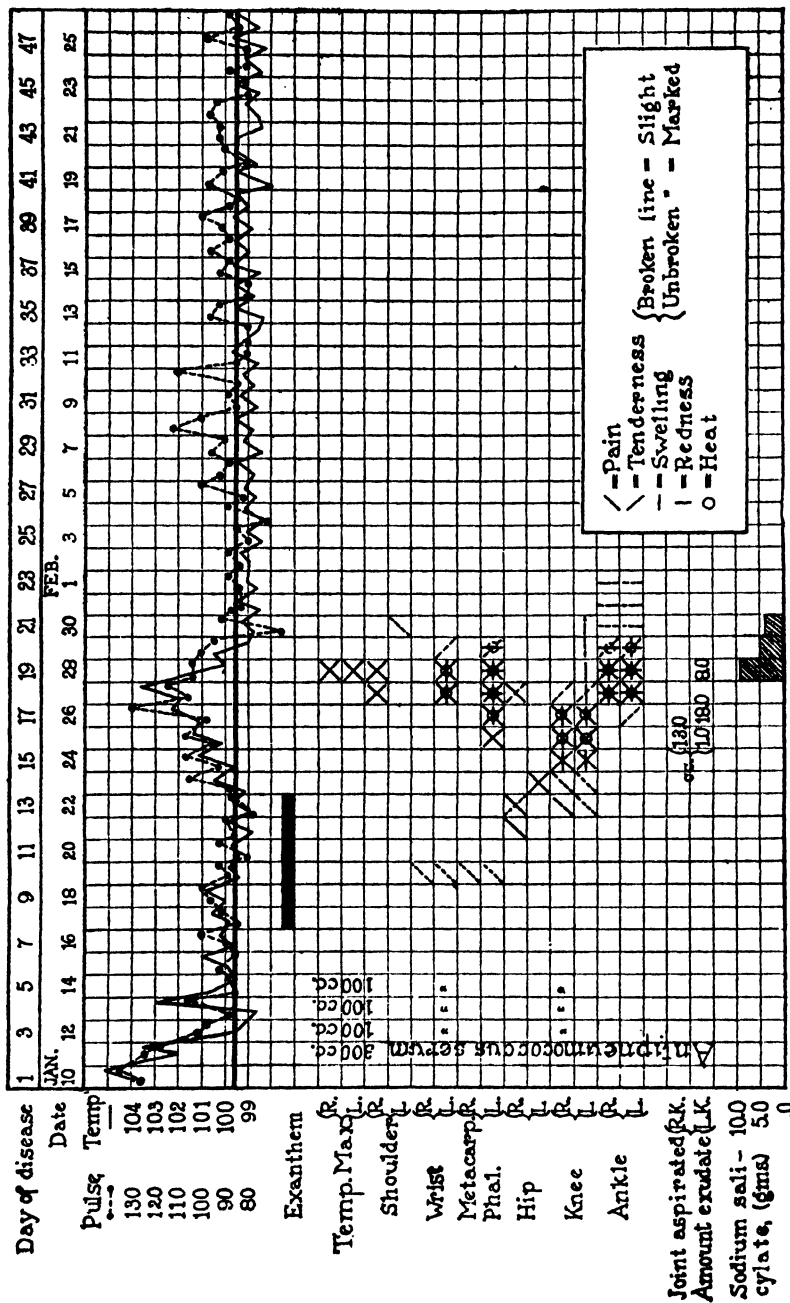


Chart 1 (Case 1). Temperature curve, exanthem and joint involvement in case of serum sickness.

The nature of the arthritis in this patient was the subject of considerable discussion. It appeared with other symptoms of serum disease and disappeared abruptly at the time a serum disease arthralgia would be expected to subside. The additional evidence of distinct amounts of antigen (horse serum) in the exudate early, at the height of the disease, and the almost complete disappearance of the horse serum from the joint two days later, when the symptoms subsided, are comparable with the findings in the blood serum of patients with serum disease. Painful temporomaxillary joints, such as this patient showed, are common in serum disease and rare in rheumatic fever. On the other hand, the intensity of the inflammation in the ankles, knees and left hand was much greater than that usually encountered in serum disease, and more like that of rheumatic fever.¹¹ The disappearance of arthritis following the saturation of the patient with sodium salicylate is another point in favor of rheumatic fever; for one of us has administered salicylates to patients with serum sickness arthritis without any beneficial effect. In this patient, however, the arthritis of serum disease would naturally be expected to subside at this time; and the concomitant disappearance of antigen from the joint fluid, noted above, led us to consider the arthritis to be the result of serum disease.

CASE 2.—N. M., a woman, aged 19, was admitted, Jan. 17, 1922, on the second day of pneumonia with consolidation of the right lower lobe. A blood culture was sterile. Pneumococci, Type I, were obtained from the sputum. During the second, third and fourth days of illness, 640 c.c. of Type I antipneumococcus horse serum (New York State Board of Health, Lot 187 A) was given intravenously; crisis occurred on the fourth day. Serum sickness began two days later. A mild arthritis occurred on the fifth day of the serum sickness, and lasted two days. Although there were only uncertain signs of an increase of fluid, the right knee joint was aspirated, and 0.5 c.c. of exudate was obtained, having a leukocyte

¹¹ At the time of correction of proof, a patient with definite serum disease is under observation. He shows not only pain and tenderness of all of the affected joints, but clinical evidence of effusion into the left knee from which 5 and 7 c.c. of turbid fluid was aspirated on the third and fourth days, respectively; 2 c.c. of fluid was also aspirated from the right knee, even though it showed no clinical evidence of effusion. The ankles showed pain, tenderness, swelling and local heat. This case seems to prove conclusively that marked evidence of inflammation may be found in the "arthritis" of serum disease.

content of 12,000 cells for each cubic millimeter, 64 per cent. of which were polymorphonuclear leukocytes. No general glandular enlargement occurred. Serum sickness lasted eight days. Convalescence was uneventful. There was no previous history of polyarthritis.

CASE 3.—E. M., a man, aged 24, was admitted, March 30, 1922, on the second day of illness, with consolidation of the left lower lobe. A blood culture was sterile; Type I pneumococci were recovered from the sputum. During the course of four days, 750 c.c. of Type I antipneumococcus serum (New York State Board of Health, 650 c.c. of Lot 196 and 100 c.c. of Lot 198) was injected intravenously, beginning the first day after admission. On the fifth day of illness the physical signs indicated an extension to the right lower lobe. Improvement occurred

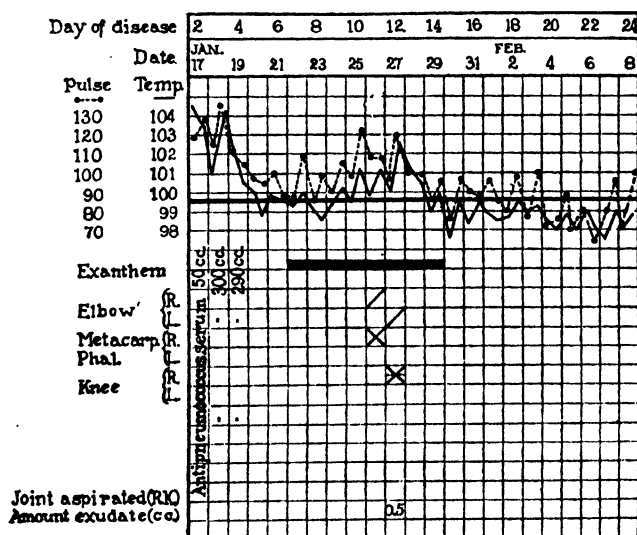


Chart 2 (Case 2). Temperature curve, exanthem and joint involvement in case of serum sickness.

two days later. Serum disease began with urticaria six days after the administration of the first dose of serum. The exanthem lasted nine days. Glandular enlargement, glossitis and multiple arthritis were present; the arthritis lasted four days. Clinically, there was evidence of fluid in the right knee joint. This was aspirated, and 2.5 c.c. of greenish yellow, faintly turbid exudate was removed. The fluid contained 4,400 leukocytes for each cubic millimeter, of which 44 per cent. were polymorphonuclear neutrophil cells. A relapse of the serum disease, consisting of urticaria, increase in temperature and pulse, glandular enlargement and mild arthralgia, occurred after a free interval of seven days. It lasted eight days and was followed by an uneventful convalescence. There was no previous history of polyarthritis.

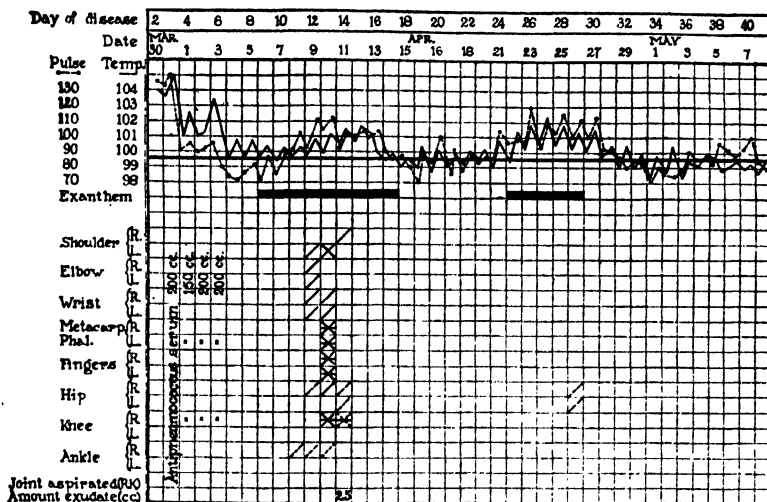


Chart 3 (Case 3). Temperature curve, exanthem and joint involvement in case of serum sickness.

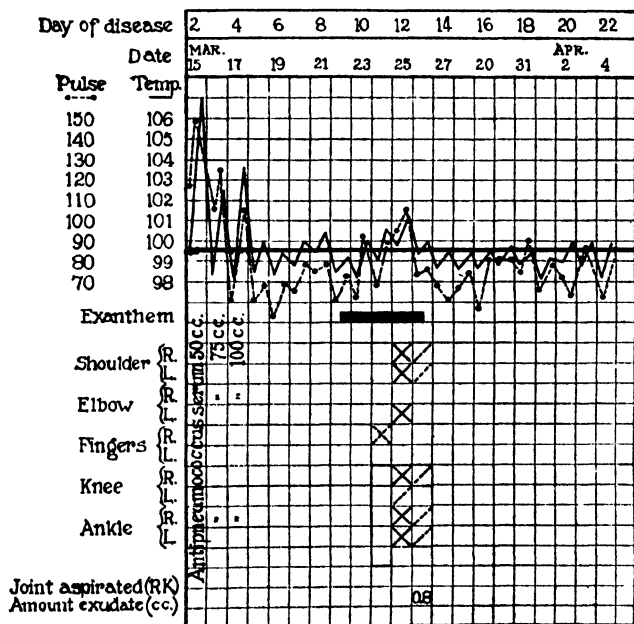


Chart 4 (Case 4). Temperature curve, exanthem and joint involvement in case of serum sickness.

CASE 4.—L. B., a boy, aged 17, was admitted, May 15, 1922, on the second day of pneumonia, moderately ill with involvement of the right lower lobe. Blood culture was negative. Type I pneumococci were found in the sputum. Over a period of three days, 225 c.c. of Type I antipneumococcus serum was given intravenously, with return of temperature to normal. On the seventh day following the first serum administration, urticaria and general glandular enlargement appeared. A moderately severe multiple joint involvement developed two days later, and disappeared completely after three days. Although no definite evidence of swelling could be determined, the right knee was aspirated, and 0.75 c.c. of turbid exudate removed. This exudate contained 12,800 leukocytes for each cubic millimeter, the majority of which were mononuclear in type. The fluid was examined for the presence of antigen (horse serum); a positive precipitin reaction occurred with antihorse rabbit serum in a dilution of 1:15. The patient had serum sickness for six days, and recovered normally. He had had an attack of acute rheumatic fever with polyarthritis one year previously.

RESULTS AND COMMENT.

In no case was arthritis evident until from two to five days after the appearance of the urticaria. The duration of symptoms in a single joint varied from a few hours to nine days. A striking point was the disappearance of arthritic symptoms in all of the joints of a patient within twenty-four hours of the subsidence of fever. Although pain and tenderness were the only signs of involvement in many of the joints, the recovery of fluids having the character of exudates indicated that the process was inflammatory in nature. Moreover, the clinical examination of the joints in Case 1 revealed the intensity of the process in certain instances. The doubtful nature of this case has already been discussed. Taken alone, it would not be very conclusive; but a gradation of intensity from very mild to very severe is seen in comparing the cases in this order: 2, 4, 3, 1. Such clinical comparison leads one to conclude that all of the arthritic symptoms were due to a common cause—i.e., serum disease.

A description of the exudates is given in the accompanying table. Only the knees were aspirated because of the difficulty in obtaining fluid from smaller joints. As only very small quantities of synovial fluid can be aspirated from normal joints, and as this fluid is partially devoid of cellular elements, it is safe to conclude that the recovery of 0.5 c.c. or more of turbid fluid constitutes definite evidence of exudation, especially as these fluids in every instance were micro-

Exudates Aspirated from Joints of Patients with Serum Sickness.

Case.	Date.	Joint.	Severity of inflammation at time of aspiration.	Quantity aspirated, Cc.	Leukocyte count.						Precipitin test for horse serum.	
					Total.	Differential.						
						Poly-morpho-nuclear neutro-phils.	Large mono-nuclears.	Small lympho-cytes.	Poly-morpho-nuclear eosino-phils.	Pink staining cells with pyknotic nuclei.*		Unclassified cells.
1	1/25/22	Right knee	Marked	13.0	22,400	86.0	9.0	2.0	...	1.0	2.0	1:1,000
	1/25/22	Left knee	Marked	1.0		78.0	16.0	4.0	...	...	2.0	1:100
	1/26/22	Left knee	Marked	18.0	14,000	84.0	10.0	3.0	...	3.0	...	1:10 ?
	1/28/22	Left knee	Swelling only	8.0	5,000	20.0	58.0	8.0	...	5.0	9.0	
2	1/27/22	Right knee	Marked	0.5	12,000	64.0	32.0	2.0	...	1.0	1.0	
3	4/11/22	Right knee	Slight	2.5	4,460	41.0	49.0	5.0	...	3.0	2.0	
4	5/26/22	Right knee	Slight	0.8	12,640	13.0	76.5	6.0	1.0	0.5	3.0	1:15

* These cells were of the same size as polymorphonuclear neutrophilic leukocytes; they had pink staining cytoplasm and pyknotic circular nuclei, single or multiple.

scopically abnormal. In general, the higher cell counts and greater proportion of polymorphonuclear leukocytes occurred in the more acutely involved joints; while the lower total counts and higher proportion of mononuclear cells were found in the fluids from joints mildly abnormal or longer inflamed.

These exudates were indistinguishable cytologically from many of those which we have recovered from inflamed joints of patients with acute rheumatic fever, intermittent hydrarthrosis, and chronic arthritis; and it is of importance to show that such exudates can be produced without the actual presence of bacteria.

The presence of horse serum in two of the joints and the constantly diminishing amount in one of them suggest a possible cause of the arthritis. It may be that in a patient whose tissues are allergic to horse serum the presence of that serum in the synovial cavities sets up an irritation in the lining membrane; and the disappearance of that serum may be the cause of the subsidence of this irritation. Although some workers have considered that the disappearance of the horse serum from the blood stream was due to the development of circulating antibodies, the more recent work of Mackenzie¹² has thrown some doubt on this opinion. In view of the uncertainty as to the relation between cause and effect in circulating antibody and antigen, it is impossible, at present, to be definite in reference to the effect of these substances in exudates.

SUMMARY.

Among ten patients with serum disease who had received large amounts of horse serum intravenously, there were six who showed definite joint involvement. From the joints of four of these it was possible to aspirate fluid having the gross and microscopic character of an exudate. From a fifth, in whom pain was the only symptom, no fluid could be obtained; the joints of the sixth were not aspirated. The positive findings in the synovial fluids from the four other patients, however, indicate that the arthritic symptoms in serum disease are due to an actual inflammation of the joints. The finding of horse

¹² Mackenzie, G. M., and Fruhbaar, E. L.: Proc. Soc. Exper. Biol. & Med. 19: 269, 1922.

serum in two fluids suggests that the irritation of the joint may be due to the presence of this foreign protein in an allergic tissue.

Clinically, the polyarthrititis of serum disease is usually manifested by pain and tenderness of the joints, but in certain instances swelling, heat and redness may be present. The synovial exudate is microscopically indistinguishable from that occurring in joints of patients with rheumatic fever.

COW SERUM AS A SUBSTITUTE FOR COLOSTRUM IN NEW-BORN CALVES.

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(Received for publication, May 31, 1922.)

In a former communication¹ it was shown that if new-born calves are divided in two groups, one of which receives colostrum from the dam and the other not, and if both groups are removed promptly from the surroundings of the herd, placed in individual stalls, and fed raw milk, the calves fed colostrum survive and develop as normal calves, while of the calves from which colostrum has been withheld the greater number die within the 1st week as a result of the invasion and multiplication of intestinal bacteria, chiefly *Bacillus coli*, throughout the body. Those that survive may develop lesions in or about certain joints, more rarely foci of sclerosis in both kidneys.

EXPERIMENTAL.

The rapid absorption into the blood of agglutinins towards *Bacillus abortus* ingested in the colostrum² indicated that the immunity of the calves receiving colostrum is due to the protective antibodies which tend to accumulate in the colostrum up to the time of parturition. If this inference is true the blood of the adult cow should also contain these various antibodies and might protect calves not fortified by colostrum. To test this hypothesis, three groups of calves were treated with the blood serum of the same cow in slightly different ways.

Calves of which it was certain that they had not suckled the dam were brought to the animal quarter of the Department soon after birth, thoroughly washed with water of about body temperature, and

¹ Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 181.

² Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.

dried by rubbing with towels. Each calf was placed in a separate stall and fed milk from cows in about the middle of the lactation period. The milk was at first fed from bottles or other receptacles through nipples and after the 1st week from pails.

The serum used throughout was obtained from the same cow at intervals during the experiment. The animal was a grade Guernsey, in milk when purchased from the Middle West November, 1920. She was chosen at random mainly because easily controlled. The blood was drawn from a jugular vein under aseptic conditions and allowed to clot. The serum was withdrawn after 2 days, passed through a Berkefeld filter, and stored at 38–40°F. without an antiseptic.

No efforts beyond isolation in individual stalls and ordinary cleanliness were made in rearing the calves. Owing to the nature of the experiment and the many uncontrolled factors entering into it, it has seemed best to record certain details which may be of significance in the interpretation of results. These are given in the notes on the individual calves.

The first group of five calves received serum both into the jugular vein and the subcutis immediately after the cleansing bath. On the following day serum was again injected into the subcutis. The following notes furnish the chief details.

Calf 901.—Guernsey bull, born Mar. 10, 1.15 p.m., of a native cow in her second pregnancy. Removed from dam immediately after birth. Received at Department 2.45 p.m. Washed in water at 100°F. containing a little soda. Dried off by gentle friction with bath towels. Calf quite weak after the cleansing. Kept warm with blankets. Received into jugular vein 20 cc. serum and 20 cc. into subcutis in three different places. 5 p.m. Fed 1½ lbs. milk. 11 p.m. Second feeding of ¾ lb. milk.

Mar. 11. Meconium discharged during the night. Calf received about 2 lbs. milk. Urine collected has a specific gravity of 1,008. Contains no abnormal products. At noon calf received into subcutis 20 cc. serum. Diarrhea started late today. Temperature normal. Total amount of milk taken about 4 lbs.

Mar. 12. Stools less fluid, light yellow. Temperature normal. Total milk taken about 6 lbs.

Mar. 13. Feces consistent, orange to yellow. 7½ lbs. of milk taken.

Mar. 16. Calf is fed from pail. Takes about 7½ lbs. of milk.

Apr. 12. Calf has progressed normally, taking slightly more milk each week. Today 12 lbs. consumed. Since Apr. 4 and 5 a little hay and grain were placed in the stall each day.

Apr. 13. Calf killed today. Weight 102 lbs. Autopsy negative with exception of oecum in which there is a wide meahed pigmentation over the submucous vessels.

Calf 538.—Holstein bull, born 12.40 p.m., Mar. 16, of a cow in her third pregnancy. Brought to Department at 1.15 p.m. Washed in the same way as described for Calf 901. 2 p.m. Fed $\frac{1}{2}$ lb. of milk. 2.15 p.m. 18 cc. of serum injected into jugular vein and 22 cc. into subcutis. Fed milk at 5 and 10.30 p.m. Calf weak and able to stand only a short time.

Mar. 17. Begins to discharge meconium during the day. Takes milk from bottle readily, about 4 lbs. in all taken today. Stronger, moves about stall. Receives 20 cc. serum into subcutis at 3.30 p.m. Late in the evening calf was quite weak, scarcely able to stand. Extremities cold. Mucous membranes pale. Able to take only a little food at 11 p.m.

Mar. 18. Dies at 2.30 a.m. Weight after death 66 lbs. The autopsy showed no gross lesions to account for death. The fourth stomach contained over a liter of milky fluid and curds. The latter made up about one-third of the total. Mucosa was sprinkled over with deep and superficial petechiæ.³ In the small intestine occasional congested areas. In the large intestine the longitudinal folds form dark red streaks of congestion and hemorrhage. Cultures containing bits of tissue from spleen, liver, and kidneys remain sterile. From several segments of the small intestine *B. coli* isolated as the chief type present.

Calf 485.—Guernsey heifer, born Mar. 24, 9.15 a.m., of a native cow in her third pregnancy. Removed immediately and brought to Department. Washed and dried. 10.27 a.m. Injected 20 cc. serum into jugular vein and 20 cc. into subcutis. Calf rather weak. 1.45 p.m. Receives the first dose of milk from bottle. Fed again at 5 and 11 p.m. Calf active. Rather dry meconium voided.

Mar. 25. Feces growing soft, liquid later in the day. In the evening the calf loses its activity and takes its milk more slowly. Feces very soft and somewhat fetid; light yellow in color. Took in all about 3 lbs. of milk.

Mar. 26. Has been scouring during the night and continues during the day. Fed five times today, the calf taking only small amounts. Very weak late at night.

Mar. 27. Calf very weak, unable to get up. Takes a few ounces of milk. Temperature 37.2°C. Extremities growing cold. Chloroformed later in the afternoon and refrigerated. Calf weight 49 lbs.

Mar. 28. Autopsy shows the following abnormalities. Fourth stomach nearly empty. Minute hemorrhages present in small number. Upper small intestine congested in streaks and patches. Fat stasis indicated by whitish club-shaped villi. Epithelium contains fat granules. The rest of small intestine deeply congested in streaks, patches, and continuously for some distance. Longitudinal streaks of congestion in large intestine. Kidneys uniformly congested. Lungs mottled with pale red patches of atelectasis.

³ Smith, T., and Little, R. B., *J. Exp. Med.*, 1922, xxxvi, 184.

Agar slant containing a bit of kidney tissue remains sterile. One plus spleen tissue develops only around the bit of tissue. Subcultures from this contain besides *B. coli* a small diplococcus (enterococcus). A tube containing a bit of liver tissue also develops growth only about the tissue. Subcultures show only *B. coli*. The same type of *B. coli* was isolated from two segments of the small intestine in pure culture. This type is characterized by marked viscosity of the growth and large involution forms. Occasional motile elements detected.

Calf 767.—Holstein heifer, first calf of native cow, born 11.15 a.m., Mar. 24. Removed at once from dam, taken to Department, washed, dried, and blanketed. 11.55 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Fed first milk at 3.15 p.m., next at 11.10 p.m. Calf, weak after cleansing, was stronger at night.

Mar. 25. Voids a large amount of dark greenish meconium. At 11 a.m. 20 cc. serum injected into subcutis. Fed three times today, in all about 3 lbs. of milk.

Mar. 26. Feces becoming softer and fluid late in the day, yellowish in color. Fed three times, in all 3½ lbs. of milk.

Mar. 27. Weaker today. Feces still fluid. Took only 3½ lbs. of milk.

Mar. 28. Calf quite weak. Feces still soft and yellowish. Takes 3½ lbs. of milk in all.

Mar. 29. Calf unable to stand. Has to be held when taking food. Fed four times. Took 3½ lbs. in all.

Mar. 30. Calf begins to improve. Feces less fluid. Takes 4 lbs. of milk. Gets up and moves about stall.

Apr. 19. Since Mar. 30 the calf has improved steadily and continued to take larger quantities of milk. Today 11 lbs. taken. The calf is emaciated and shows stiffness, without localized joint lesions, in the hind limbs. For several days (Mar. 30 and 31) there was a slight conjunctivitis.

Apr. 20. Calf killed today. Weight 78 lbs. No lesions were found. Cultures made with bits of spleen, liver, kidneys, and several drops of fluid from both knee and tarsal joints remained sterile.

Calf 948.—Female Holstein calf, born of a heifer in her first pregnancy, at 1.30 p.m., May 3. Calf weighs 88½ lbs. Labor difficult and calf delivered in the last stage by traction. Removed at 3.50 p.m. to the Department, washed, and dried. 4.40 p.m. 40 cc. serum injected into jugular veins and 20 cc. into subcutis. 7.50 p.m. First meal of 2 lbs. of milk. Fed again at 10.30 p.m. and 1 a.m.

May 4. Receives into subcutis 40 cc. serum. Fed four times today, the last meal at 10 p.m. Drank in all about 8 lbs. of milk.

May 5. Feces becoming soft and observed to be very watery and ejected in a stream at 1.15 p.m. Calf weak, growing thin. Eyes sunken. Has taken only about 6 lbs. of milk today.

May 6. Calf weaker; still able to stand but gait unsteady. Feces fluid during the day, light yellow in color. Takes in all about 4 lbs. of milk.

May 7. Temperature is 40.2°C. this morning. Feces continue fluid. Calf unable to stand. Takes but little milk when fed at 11 a.m. 48 cc. of cow serum injected subcutaneously. Calf dies this afternoon. Body chilled with cold water and refrigerated till next morning.

May 8. From the autopsy notes the following may be mentioned. The fourth stomach contains a small amount of a thick, viscid material plus small curds. Hemorrhagic blotches scattered through leafy portion. In the small intestine there is moderate congestion in patches above which increases in intensity downwards. In the ileum the mucosa is dark red and overlaid with a surface film of orange-colored mucoid material suggesting superficial necrosis. The large intestine is congested along summits of the longitudinal folds. Films from small intestine contain many rods, like *B. coli*.

Bits of spleen and kidney in agar slants remain sterile. In the liver tube one colony of *B. coli* appears. The dilution cultures from the small intestine contain colonies of *B. coli* only.

It will be noted that two of the five calves died in about 48 hours after birth. A third was chloroformed, moribund, when 3½ days old. A fourth calf was very sick when 3 days old, but recovered. The fifth calf passed through a period of diarrhea lasting nearly 2 days, but continued to take its food and made a good recovery.

Of the three calves that died, the organs of one were sterile. In the second case, the kidneys were sterile but growth appeared about the bit of liver and spleen tissue, indicating the presence of very few bacteria as compared with the condition of the same organs in controls not receiving either colostrum or serum. In the third both spleen and kidneys were sterile and the liver tube contained one colony.

A second group of five calves received the bovine serum not only intravenously and subcutaneously but also in the milk of the first two meals. The following notes of these cases supply the individual data.

Calf 768.—Holstein bull, born 5.45 p.m., Apr. 10, of a native cow in her third pregnancy. Weighs between 85 and 90 lbs. Removed immediately to the Department, washed, and dried. 7.20 p.m. 20 cc. serum injected into subcutis and 20 cc. into jugular vein. 10.10 p.m. Fed nearly 2 lbs. of milk plus 60 cc. serum mixed with it. 2.30 a.m. Fed milk and 60 cc. serum.

Apr. 11. Considerable meconium passed during the night. 20 cc. serum injected subcutaneously at 9.45 a.m. Takes in all 8 lbs. of milk today.

Apr. 12. Diarrhea during night and this morning. Takes food as usual.

Apr. 13. Feces soft and grayish yellow. Calf takes 8 lbs. of milk today.

Apr. 30. The calf has been progressing normally since the preceding date. Took 11 lbs. of milk today.

May 1. Killed and found normal.

Calf 905.—Holstein bull, born Apr. 7, at 10.30 a.m., of a native cow in her fourth pregnancy. Weighs 114 lbs. Taken to the Department immediately after birth, washed, and dried. 11.40 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into subcutis. 2 p.m. Received first meal of milk, 1½ lbs. plus 60 cc. serum. 5.40 p.m. Fed the same quantity of milk and serum. 10.35 p.m. Fed 2 lbs. of milk.

Apr. 8. Receives 20 cc. serum into subcutis at 10 a.m. Considerable dark greenish meconium discharged today. Feces soft late at night.

Apr. 9. Feces still fluid. Considerable blood discharged at one time during defecation. Calf otherwise active and hungry. Takes in all today 9 lbs. of milk.

Apr. 11. Feces still slightly tinged with blood.

Apr. 13. Takes in all 11 lbs. of milk. Lameness in right fore leg without local signs.

Apr. 14. Lame in right hind leg. Hock and ankle swollen. Apr. 15. Temperature slightly elevated (39.6°C.). Blood still voided on feces. Apr. 17. Stiffness and lameness have disappeared. Calf very active. Apr. 23. Takes 12 lbs. milk. Apr. 28. Slight febrile disturbance. Apr. 29. Normal today.

May 1. Killed for food. Organs normal.

Calf 906.—Holstein female, born 9.30 a.m., Apr. 11, first calf of a native heifer. Calf small, weighing 65 lbs. Received at Department at 10.30 a.m., washed, and dried. At 11.10 a.m. 20 cc. serum injected into a jugular vein and 20 cc. into the subcutis. 2 p.m. Fed 2 lbs. of milk to which were added 60 cc. serum. 5.35 p.m. The same dose repeated. 10.30 p.m. Fed 2 lbs. of milk.

Apr. 12. Receives into subcutis 20 cc. serum. Meconium passed during the night. Calf active and takes its food well.

Apr. 13. Feces liquid, greenish yellow. Calf otherwise normal. Apr. 15. Took 6½ lbs. of milk today. Apr. 18. Took 8 lbs. of milk today. Apr. 21. Took 11 lbs. of milk today.

May 1. Killed for food. Organs normal.

Calf 932.—Holstein bull, born of a western cow, at 7.20 a.m., Apr. 17. Calf weighs 100 lbs. Taken to Department, and washed and dried. 10 a.m. Receives 20 cc. serum into a jugular vein and 20 cc. into the subcutis. Refuses food at noon. Takes 2 lbs. of milk at 4.50 p.m. to which 60 cc. serum had been added. At 10.15 p.m. this dose was repeated.

Apr. 18. Receives 20 cc. serum into subcutis. Calf constipated.

Apr. 19. Feces fluid, light yellow in color, later in day nearly colorless. Takes milk normally.

Apr. 20. Feces still soft.

Apr. 21. Takes 9 lbs. of milk in all. Some blood discharged on feces.

Apr. 24. Takes 11 lbs. of milk. Appears entirely normal. Apr. 26. Took 11 lbs. of milk today. Some blood passed on feces. Apr. 27. Blood still passed with feces. Apr. 29. Feces normal.

May 1. Killed for food. Normal.

Calf 953.—Guernsey female, born May 20, 9.15 a.m., of a cow in her fourth pregnancy. Calf rather thin, weighs 70 lbs. Received at the Department at 10.15 a.m. Simply dried off, without preliminary washing, with bath towels. Calf able to stand and move about. 10.40 a.m. Received into jugular vein about 39½ cc. of serum and 20 cc. into subcutis. 11 a.m. Fed 2 lbs. of milk plus 55 cc. serum. 4.20 p.m. The same feeding repeated. 11.15 p.m. Fed 2 lbs. of milk.

May 21. Calf very eager for food. 2 lbs. of milk taken. 9.30 a.m. 20 cc. serum injected into subcutis. Urine collected at this time. No fluid feces observed today. Fed in all 6 lbs. of milk.

May 22. Took 6½ lbs. of milk today. Feces passed resemble meconium. No diarrhea.

May 26. Slight diarrhea today. For several days past, the feces have been rather light in color but of normal consistency. Today the calf took 8 lbs. of milk.

May 27. Feces slightly softer than normal, light yellow. Urine collected early shows 0.06 per cent albumin (Esbach), specific gravity 1.010, slightly acid, pale yellowish, and faintly clouded. Sediment slight, containing some short hyaline casts filled with amorphous granules.

May 28. Feces normal. Only a trace of albumin in urine today.

May 30. Normal.

Two of these five calves manifested a slight diarrhea associated with hemorrhage which soon disappeared. Four were killed when 14, 21, 24, and 20 days old respectively. No abnormalities were noted. The fifth is still alive.

A third group of five calves received the serum in the milk of the first and second meals. Whatever protection was afforded by it had to be exerted through the digestive tract.

Calf 772.—Small Guernsey calf, born at 12.10 p.m., Apr. 19, of a native heifer in her first pregnancy. Weight 50 lbs. Taken away before it had suckled the dam, received and cleansed at 2 p.m. Calf very weak, unable to stand. Large amount of dark, soft meconium voided. 4.45 p.m. First feeding of 1 lb. of milk to which 100 cc. of serum had been added. 10.30 p.m. Calf fed 1½ lbs. of milk to which 100 cc. of serum had been added. Still unable to get up.

Apr. 20. Calf continues weak. Fed milk at four intervals during the day. During the last feeding at 10.15 p.m. the calf failed to take its food. Meconium voided during the day, now yellowish.

Apr. 21. Calf died at 4 a.m. Considerable diarrhea preceding death. Autopsy next morning presents the following abnormalities.

Fourth stomach contains a handful of white, rather firm curds and some milky fluid. Mucosa normal. Mucosa of small intestine deeply congested throughout. Large intestine less so.

Endocardium of left ventricle raised in the form of linear ridges by subendocardial hemorrhages.

Kidneys deeply and uniformly congested throughout. Urine taken from bladder shows only a trace of clouding when slightly acidified with acetic acid and heated to boiling.

Cultures prepared by adding bits of tissue from spleen, liver, and kidneys to slanted agar remained sterile. Cultures from successive dilutions of contents of small intestine contain colonies of *B. coli* and some colonies of smaller size, probably enterococci.

Calf 935.—Holstein female, weighing about 85 lbs., born 6.30 a.m., Apr. 21, of a heifer. Calf not allowed to suckle dam. Received at Department at 9.40 a.m., washed, dried, and blanketed. 11.40 a.m. Received first meal of 2 lbs. of milk plus 100 cc. serum. 4.30 p.m. The same dose repeated. 10.20 p.m. Fed 2 lbs. of milk.

Apr. 22. Dark meconium passed during night. Feces grew liquid towards noon, yellowish in color. Calf took 4½ lbs. of milk in all today.

Apr. 23. Scouring a little early in the day. No movements later. Fed four times and took about 7 lbs. of milk.

Apr. 24. Calf improving and quite active. Fed three times, in all about 7 lbs. of milk. Urine caught is normal, specific gravity 1.012.

May 15. Has been taking 15 lbs. of milk daily. In good condition. Sold to butcher today. The organs appear normal with exception of kidneys. On close scrutiny there are seen in many of the lobes groups of feebly accentuated grayish areas less than 1 mm. in diameter. On section these correspond to narrowly triangular pale areas passing radially to medulla.

Calf 936.—Large Holstein bull, weighing about 100 lbs., born 9.20 a.m., Apr. 21, of a heifer (first pregnancy). Owing to its large size calf had to be delivered by traction in the last stage. Calf kept from suckling dam and taken to Department at 11 a.m., washed, dried, and blanketed. Milk refused by calf until 4.35 p.m. when it took 1½ lbs. plus 100 cc. serum. A little of this mixture was lost in feeding. 10 p.m. Fed about 2 lbs. of milk plus 100 cc. serum. The calf has been rather weak all day.

Apr. 22. Large amount of meconium passed during the night. Feces becoming fluid during the day. Takes but little milk.

Apr. 23. Calf more active and feces firmer, yellowish in color. Fed four times, taking in all about 8 lbs. of milk.

Apr. 24. Calf active. Feces still soft and light yellowish in color.

Apr. 29. Slight swelling and lameness of left tarsal joint. Left fore leg shows lameness but no recognizable swelling. Receiving 12 lbs. of milk.

May 13. Calf not quite normal. Drank milk slowly. Evening temperature 39.9°C.

May 14. Condition unchanged. Takes 12 lbs. of milk. Temperature 39°, 39.8°, and 40°C.

May 15. Temperature 40°C. Killed today. The only abnormality found is in the kidneys. Multiple foci varying from 2 to 8 mm. in diameter; from two to twelve in a lobe. These are firm, smooth, and glistening white on section. They are broadly triangular with base at the surface and apex at junction of cortex with medulla. No reactive zone around them.

Calf 940.—Large Holstein heifer, weighing about 110 lbs., born 2.20 p.m., Apr. 25, of a native cow in her second pregnancy. Treated in the usual way. Calf weak and unable to stand. 4.40 p.m. Received the first food, 2 lbs. milk plus 100 cc. serum. 11 p.m. Fed 2½ lbs. milk plus 100 cc. serum. Dry meconium discharged.

Apr. 26. Feces became fluid towards afternoon, light yellow in color. Fed four times today. Took in all 8 lbs. of milk.

Apr. 27. Diarrhea checked. Calf active and hungry.

Apr. 29. Takes 11 lbs. of milk.

May 3. Takes 14 lbs. of milk.

May 15. This calf has been normal, taking 17 lbs. of milk since May 11. Killed today. Organs normal.

Calf 951.—Holstein female, born May 8, at 4.30 p.m., of a heifer. Parturition normal. Calf weighs 77 lbs., rather thin. Taken to the Department at 5 p.m., washed, dried, and removed to a single stall. 7.10 p.m. Fed the first meal of milk plus 100 cc. cow serum. 11.40 p.m. Received 2 lbs. of milk plus 120 cc. serum. Moves actively about stall.

May 9. Fed 2 lbs. of milk plus 120 cc. serum. Calf takes the mixture readily. This is the last serum fed. Milk taken at 5 and 10 p.m. Calf has been constipated. Meconium passed during the night.

May 10, 8.15 a.m. Temperature slightly elevated (39.4°C.). Diarrhea sets in. Calf feeds slowly and shows signs of depression. Grows weaker during the day while diarrhea continues. Feces tinged with blood. Temperature at 11 p.m. 39.6°C.

May 11. Calf dies at 5 a.m. Chilled with cold water and refrigerated until 9.30 a.m. Of the autopsy notes, the following are significant. Fourth stomach contains a small amount of milky fluid and some curds, totalling a mass 4 to 5 cm. in diameter. Mucosa covered with a thin layer of mucus. Minute hemorrhagic points, deep, faded, and superficial appear in two dense groups covering a total area about 10 cm. in diameter. Small intestine congested throughout in streaks and patches, the congestion increasing downwards. Large intestine congested along longitudinal folds.

Medulla of kidneys deeply congested. Bladder distended with 775 cc. urine. No obstruction found to account for the retention. Specific gravity of urine 1,010. Faintest trace of albumin present.

Marked fatty degeneration of liver cells. Orange pigment granules present.

Bits of liver and kidney in agar tubes remain sterile. In the spleen tube in place of *B. coli* about fifteen colonies of an enterococcus present. *B. coli* appears in cultures from the small intestine as in earlier cases.

It will be noted that two of this group died. One was small and weak from the start, unable to get up, and lived less than 2 days. The spleen, liver, and kidneys were sterile, however. Since the organs of all control calves thus far contained numerous bacteria, it may be assumed that the serum was responsible for this condition.

The second calf which died lived $2\frac{1}{2}$ days. The liver and kidneys were sterile but in the spleen tube about fifteen colonies of enterococcus appeared.

Since in the former series¹ such a high percentage of calves from which colostrum had been withheld died, only two additional calves were treated in a similar way as controls during the period of this experiment. These died within 2 days. Spleen, liver, and kidneys were flooded with bacilli of the *Bacillus coli* type.

Calf 945.—Female, born of a native heifer Apr. 30, 10.20 a.m. Weighs 79½ lbs. Not allowed to suckle the dam and taken to the Department buildings at 10.40 a.m. Washed and dried and removed to a single stall. Calf fed at 1 p.m. Drinks with difficulty and takes only 10 ounces. 6 p.m. Takes about 1 lb. of milk very slowly. Fed again about 1½ lbs. at 10 p.m. During the day considerable meconium was discharged.

May 1. Takes about 8 lbs. of milk during the day. Bright and active early in the day. Feces become watery later in the morning and continue so during the day. Later tinged with blood.

May 2. Calf died during the night. Autopsied early this morning. Externally nothing abnormal. The fourth stomach somewhat dilated. It contains several firm white curds, the size of an egg, and considerable milky fluid. The entire mucosa has a deep red color. In the small intestine the mucosa is diffusely and deeply congested in the middle portion, in upper and lower segments less so. Films show many bacilli resembling *B. coli*.

Beneath endocardium of left ventricle there are many hemorrhages. The blood in general is in soft, dark clots, filling the large vessels with large molds. The liver contains much fat and orange pigment. The kidneys are uniformly congested to the tips of papillæ. Urine from bladder contains a trace of albumin; clear, yellow, specific gravity 1.032.

Films from spleen, liver, and kidney tissue contain scattering colon-like rods in every field. Cultures from these organs indicate presence of large numbers of bacteria. Only after one dilution of a bit of tissue in 6 cc. of bouillon are colonies

discrete on agar slants. The bacteria resemble *B. coli*. Dilution cultures from a segment of the middle zone of the small intestine show *B. coli* and enterococci.

Calf 947.—Holstein bull calf, delivered by mechanical help, May 3, 10 a.m. Presentation normal. Mother a native heifer. Weight of calf 86 lbs. Taken to Department at 11.50 a.m. and treated as the others. It was noted when cleansing calf that the umbilical cord had been torn away during delivery. Raw surface treated with boric acid. 2 p.m. Received first meal of 2 lbs. of mixed milk. Fed again at 5.20 and 10.20 p.m. Received in all 6 lbs. of milk.

May 4. Calf's feces becoming fluid. Calf growing dull. Respirations rapid and superficial. Refuses midday meal. Fed at 5 and 10 p.m. Calf growing weaker, takes but little food. Discharges continue watery.

May 5. Feeding continued at intervals during the night and day. Symptoms as yesterday. Calf grows weaker, takes but little food. Dies towards evening. Refrigerated until next morning.

The autopsy showed few differences between this case and Calf 945. There were many superficial hemorrhagic points in the fourth stomach. The small intestine and the kidneys showed very little congestion. The liver contained considerable fat and orange pigment. The bacteriology of the organs and the small intestine was almost identical with that of Calf 945 and a description of the cultures is therefore superfluous.

During this same period two calves were placed under observation which had been permitted to take colostrum. One of these died after 5½ days. This was the first colostrum-fed calf to die in our hands. The other developed normally after a short period of diarrhea. The dead calf's organs were sterile.

Calf 949.—Holstein bull calf, weighing 99 lbs., born of a native heifer, May 3, 5.15 p.m. Labor prolonged from 11 a.m. Presentation normal. Traction applied to fore legs in the last stage. Calf allowed to suckle dam for 15 minutes, then removed to Department, washed, dried, and bedded in clean stall.

May 4. Fed 8 lbs. of milk in four doses. Calf constipated.

May 5, 10.30 a.m. Large amount of meconium and yellowish feces passed. Diarrhea started in afternoon. Respirations accelerated. Temperature 39.6°C. at 5 p.m.

May 6. Feces continue fluid. Light yellow in color. Calf appears sick. Temperature 40.1°C. at 10 p.m. Has been taking food regularly until night.

May 7. Diarrhea continues through the day. Calf growing thin. Temperature 40.2°C. at night. Took about 7 lbs. of milk today.

May 8. Calf growing weaker and unable to stand up and swallow its food late at night. Diarrhea continues.

May 9. Calf died at 4.30 a.m. Refrigerated until 9.30 a.m. Weight 76 lbs. From the autopsy notes, the following conditions are noteworthy.

The fourth stomach was distended with about 2 liters of a milky fluid and few curds. The mucosa was overlaid with mucus and congested. The small intestine was slightly congested above. The congestion increased downward and was severe in the ileum. The large intestine was congested along summits of longitudinal folds. The other viscera showed nothing unusual.

Tubes of agar containing bits of spleen, liver, and kidney remain free from growth. In dilution cultures of the middle region of the small intestine, *B. coli* was present as in preceding cases.

Calf 769.—Holstein bull calf, born Mar. 29, 8.15 a.m., of a native cow in her second pregnancy. Calf weighs about 100 lbs. 10.30 a.m. Placed with dam and allowed to suckle. Removed to Department at 1.30 p.m., washed, dried, and placed in a fresh stall. The cord had been torn off close to the abdominal wall. The exposed area dusted with boric acid powder. Calf appears strong and able to move about. Fed at 10.30 p.m. 1½ lbs. of milk.

Mar. 30. Feces become fluid during the day. Calf active. Takes in all 5½ lbs. of milk.

Mar. 31. Feces fluid and yellowish in color early, becoming less watery later in day. Milk as yesterday.

Apr. 1. Feces normal today. Calf takes 6 lbs. of milk.

Apr. 26. Calf has been normal in condition to date. Now taking 11 lbs. of milk.

Apr. 27. Killed today. No abnormalities found at autopsy.

During this same period of 2 months, twelve calves died of spontaneous scours in the herd from which our animals were taken. Two of these were autopsied.

Calf 904.—Born Mar. 31, of a native cow in her second pregnancy. Calf appeared well on Apr. 1. Diarrhea started Apr. 2. The calf grew weaker and died at noon Apr. 3. The autopsy brought out the following conditions.

Rumen contains a handful of shavings and some opalescent fluid. Similar fluid in fourth stomach totalling about 400 cc. No signs of milk or milk curds in the stomachs. The upper third of the small intestine is markedly congested, similarly the lowest third. Middle third hemorrhagic. The hemorrhages tend to raise the mucous surface into elevated streaks and patches. The large intestine is congested throughout in the form of close longitudinal lines. In the rectum the congestion involves the entire mucosa. Liver cells contain much fat. Sub-endocardial hemorrhages about papillary muscles of left ventricle.

Cultures show large numbers of *B. coli* in spleen, liver, and kidneys.

Calf 934.—Fourth calf of a native cow. Born Apr. 19. Died Apr. 20 at noon. The autopsy showed plenty of colostrum in the fourth stomach. Mucosa a uniform dark red. Small intestine is only moderately congested. There is marked fat stasis indicated by expanded, rather rigid villi full of fat. Urine taken from bladder clear, yellow, contains 0.3 per cent albumin (Esbach).

In this case spleen, liver, and kidneys are sterile. *B. coli* of the usual type isolated from contents of small intestine.

DISCUSSION.

The immediate object of the foregoing experiments was to determine whether colostrum was primarily protective against intestinal bacteria by replacing it by some easily absorbed substance containing the immune bodies found in colostrum. The substance chosen was cow serum.

If the results obtained for and against this assumption had been fairly close they could scarcely be regarded as of any value owing to the number of unknown and largely uncontrollable factors entering into such relatively crude tests. The results point, however, very decidedly in one direction—towards serum as a protective substitute for colostrum. In the first group of five calves receiving serum into a vein and the subcutis, three out of five died and a fourth was very sick. In the second group of five which received the same treatment plus serum fed in the milk none died. In the third group of five which received serum in the milk, two died.

In Table I are brought together certain data bearing on the milk fed and the serum administered. It will be seen that the milk came from three cows. In one case the 1st day's milk came from two other cows. No evidence can be adduced that the milk was responsible for the outcome. The same is true of the serum. The same cow was bled three times and the three lots of serum are designated A, B, and C. The particular lot and its age, counting from the day it was drawn from the cow, are recorded in the table. The age of the serum does not seem to figure in the results obtained. Among the group to which serum was fed, the two that died received the freshest serum of the entire lot.

The calves came from grade stock. Neither breed nor sex nor the number of the pregnancy can be drawn in as having any direct bearing on the outcome.

The results indicate that the cow serum introduced both by way of the blood and the digestive tract replaced colostrum successfully. Simply feeding the serum, though only partially successful, appears superior to injecting it. These figures become more favorable to the

TABLE I.
Calves Treated with Cow Serum in Place of Colostrum.

No.	Breed* and sex.	No. of pregnancy.	Date of birth.	Died. Age. days	Killed. Age. days	Cow's milk fed.	Age of serum used. days	Mode of administration of serum.
901	G.; male.	2nd	Mar. 10		34	678 + 712	A-22	Injected.
538	H.; female.	3rd	" 16	1½		678 + 712	" 28	"
485	G.; "	3rd	" 24	3½		678 + 712	" 36	"
767	H.; "	1st	" 24		27	678 + 712	" 36	"
948	" "	1st	May 3	4		712 + 938	B-22	"
905	" male.	4th	Apr. 7		24	678 + 712	A-50	" and fed.
768	" "	2nd	" 10		21	600 + 897	" 53	" "
906	" female.	1st	" 11		20	678 + 712	" 54	" "
932	" male.	1st	" 17		14	678 + 712	" 60	" "
953	G.; female.	4th	May 20		Normal May 30.	712 + 938	C-19	" "
772	" male.	1st	Apr. 19	1½		678 + 712	B-7	Fed.
935	H.; female.	1st	" 21		23	678 + 712	" 9	"
936	" male.	1st	" 21		23	712 + 938	" 9	"
940	" female.	2nd	" 25		19	712 + 938	" 13	"
951	" "	1st	May 8	2½		712 + 938	C-7	"

* G. stands for Guernsey, H. for Holstein.

serum treatment if we take into account certain facts. Though placed with their respective groups, one calf (No. 948) of the first group and one (No. 951) of the third group passed through the test considerably later than the others. With these was a colostrum-fed calf (No. 949) as a control. These all died. Some undetermined factors at this time increased the virulence of scours or reduced the resistance of the three calves, since this control calf is the only one of thirteen colostrum-fed calves to die. The significance of the data will be more impressive if we review the main facts of the preceding article.¹

When colostrum is withheld the body becomes flooded with *Bacillus coli* types. Other forms, such as enterococcus, also appear but they are overgrown in cultures by the more numerous and very vigorous *Bacillus coli* types. After a calf has ingested colostrum or has been treated with serum, the invasion of the body is suppressed. The digestive tract, however, may not have been protected sufficiently to prevent scours or acute diarrhea from appearing after 1 or 2 days. In fatal cases the spleen, liver, and kidneys are shown to be free from bacteria or nearly so. An examination of notes made during the past 5 years on fatal spontaneous scours in colostrum-fed calves shows that with rare exceptions in these cases also there is no *Bacillus coli* invasion of the tissues. In the few animals in which *Bacillus coli* was abundant in such organs as the spleen, liver, and kidneys, the autopsy notes showed that the calf had not suckled the dam at all.

In those cases of fatal scours in which colostrum was found in the fourth stomach there was either incomplete protection of the intestinal tract because the colostrum was ingested late or else the digestive mechanism itself was imperfect.

Among the serum-treated calves which survived and were kept 2 to 8 weeks before being killed, joint lesions were practically absent. Occasional transient lameness was noted which may have been due to temporary localization of bacteria or injury. In two of the calves (Nos. 935 and 936) which received serum in the milk focal interstitial nephritis was found when they were killed at the age of 23 days.

CONCLUSIONS.

The serum of a normal lactating cow when injected into calves a few hours after birth saved only two out of five calves so treated.

Serum added to the milk of the first two meals saved three out of five. When the two methods were combined and the serum was both injected and fed all five calves so treated survived as normal calves. These figures to be significant should be compared with the controls of both series.¹ Since the beginning of this investigation twelve out of thirteen colostrum-fed calves have survived and only four out of fifteen from which colostrum has been withheld.

In those that died the serum whether fed or injected protected the internal organs against the invasion and multiplication of *Bacillus coli* and other intestinal types and in this respect its protective action is equivalent to that of colostrum in those calves which die of spontaneous scours.

BRANCHED TUBULAR AND FLASK-SHAPED INTESTINAL GLANDS IN SOME DOMESTIC ANIMALS.*

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PLATES 1 to 5.

The histogenesis of the lymphoid tissue of the intestine has been a frequent subject of investigation, yet the glands, commonly found in the follicles situated in the region of the ileocecal valve and in the colon of some young animals, have received comparatively little notice. It is the purpose of the present paper to discuss these glands in calves and young pigs.

Historical.

The earliest mention of such glands is to be found in two papers published in 1873 on dysentery in man. In the submucosa of the intestine Cornil ('73) found cysts, filled with mucus and communicating with the lumen by a narrow neck which passed through the muscularis mucosae, and interpreted them as pathological structures resulting from the degeneration of the follicles. Kelsch ('73) also found the cysts, but interpreted them as originating from the intestinal glands of Lieberkühn which had extended into degenerating follicles. A few years later a detailed description of these glands in pigs was published by Klein ('77-'78) in his study of pig typhoid, and he named them 'flask-shaped glands.' In the same year Osler ('78) mentioned them in a short paper on pig typhoid.

During the next twenty years there appeared a number of papers on the origin of the intestinal lymph follicles and on their relation to the intestinal glands of Lieberkühn. Most of the writers were concerned with the controversy as to the source of the lymphocytes, and in some cases did not interpret the extension of the intestinal glands of

* Author's abstract of this paper issued by the bibliographic service, October 2, 1922.

Lieberkühn into the follicles of the submucosa as glands. A good résumé of this work is given by Oppel ('97). Such crypts were found in the follicles in cats by Stöhr ('89); in guinea-pigs by von Davidoff ('87), Retterer ('91, '92), Tomarkin ('93), Küchenmeister ('95), and Stöhr ('98); in rabbits by Retterer ('91), Czermack ('93), and Küchenmeister ('95); in the fetus of the horse, cow, and sheep by Retterer ('92, '95); in the duck-mole by Klaatsch ('93), and in the appendix of man by Rüclinger ('91). Orth ('01) also found submucous crypts in man, in young and old of both sexes, and in sound and in diseased intestines.

The lymphoid tissue in the intestine of domestic animals forms the subject of three dissertations that were published in Germany between 1903 and 1910. May ('03) discussed its distribution and macroscopic appearance. Schumann ('07) and Martin ('10) investigated the microscopic anatomy, but only the first-named author described in detail the pits or glands in the ileocecal valve of swine, and neither mentioned the existence of flask-shaped glands in the follicles of the colon. In 1904 Lunghetti published in Italy a short paper describing intestinal tonsils which he had found in the intestine of pigs and oxen in the neighborhood of the ileocecal valve.

The embryology of the intestine of the pig was studied by Voigt ('99), who found no structures resembling flask-shaped glands, and by Lewis, and Thyng ('08), who found diverticula. The diverticula were found especially in the distal part of the small intestine and were more numerous in the older embryos studied. Only in the oldest pig examined were diverticula found in the large intestine near the ileocecal valve, and the authors concluded not only that they might possibly develop there in later stages, but also, that "the distal diverticula probably correspond with the 'flask-shaped glands' (Klein) or 'primary submucous glands' (Stöhr) which become surrounded by lymphoid tissue and nodules."

Joest and Liebrecht ('10) made a histological and bacteriological study of the nature of the cheesy plug so often present in glands in the ileocecal valve of pigs, and in this connection described the microscopic anatomy of the glands. Also in 1910 the glands of the colon in pigs were mentioned and figured by Theobald Smith ('10), who found the lesions of the large intestine in cases of intestinal amoebiasis

of the pig to be closely associated with these glands. Again in 1915 he described and figured flask-shaped glands in the ceca of turkeys. Muthmann ('13) made a survey of the intestinal lymphoid tissue of a large number of mammals and birds, and in many cases found the intestinal glands of Lieberkühn extending into the follicles of the submucosa.

Macroscopic Anatomy.

In each species of animal there is a typical arrangement of the intestinal lymphoid tissue, and in the large intestine glands have been found in many instances associated with the follicles. In swine lymph follicles are present on the dorsal side of the ileocecal valve and in a more or less oblong patch just below the valve. In one case of an animal, killed when eleven months old, three distinct patches of follicles, approximately equal in size and lying in linear succession, were found in this region. The follicles in the colon are solitary and scattered throughout its length. They are more numerous in the upper three-fourths of the colon than in the lowest fourth, are raised somewhat above the surrounding surface, and have a diameter of 2 to 3 mm. In calves there is a similar arrangement of lymphoid tissue and glands on the ileocecal valve and in the colon immediately below it, but there the resemblance ends. Just below the second bend in the colon and where the lumen begins to narrow there is a clearly defined patch of follicles and glands, which surrounds the whole intestine and varies in size with the age of the calf. In this region the furrows of the mucosa are very marked and the wall of the intestine somewhat thickened. There are no solitary follicles in the colon of the calf.

The pits or glands on the ileocecal valve of pigs are conspicuous, and in many instances filled with protruding plugs of a cheesy consistency which can be squeezed out with the fingers. This region has been figured by Joest and Liebrecht ('10), by Ellenberger and Baum (15), and by Joest ('19), and has been named by a number of writers the 'Darmtonsille.' The orifices of the glands within the solitary follicles are much smaller, and cheesy plugs are often present in these in young animals. In calves the ileocecal valve is less prominent than in pigs and the pits are much less noticeable. Only rarely have we found them plugged as in swine. Also in both patches of follicles in the colon

the openings of the glands are very inconspicuous and in the lower patch are frequently invisible to the naked eye.

Although the lymphoid tissue in the ileocecal valve is a continuation of the aggregated follicles of the lower ileum, glands are very rarely present in any of the follicles of the small intestine. We have, however, found glands filled with plugs in this region in a few of the calves studied (Fig. 9).

Microscopic Anatomy.

The structure and disposition of the glands have been studied in series of sections made from many young animals. The routine method has been fixation in Zenker's fluid followed by staining with methylene blue and eosin. Tissues were also fixed in Bouin's and in Flemming's fluid, and other stains used were hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin, Mallory's anilin-blue connective-tissue stain, Mayer's muchematein, and Heidenhain's iron hematoxylin.

The glands have a somewhat different contour according to their location. Those in the ileocecal valve have wide orifices, which may be equal in diameter to the deepest part of the gland; so, if cheesy plugs be present, they are very conspicuous; those in the proximal patch in the colon have somewhat smaller orifices which are frequently funnel-shaped, and those in the distal patch or in solitary follicles have small circular orifices approximately 0.5 to 2 mm. in diameter. The fundi of the glands lie in the submucosa and are surrounded by an envelope of lymphocytes.

In sections through the glands in the ileocecal valve in pigs we have found the same conditions as were described by Joest and Liebrecht ('10). In the mucosa there are a number of pit-like invaginations which pass through the muscularis mucosae and penetrate more or less deeply into the lymph follicles lying in the submucosa. These pits are lined with columnar epithelium, which is continuous with that of the surface mucosa, and from their lateral borders many secondary branches are given off. These also are lined with columnar epithelium, and the whole structure resembles a much enlarged and branched intestinal gland of Lieberkühn. The epithelium is interspersed with many goblet cells, which are most numerous in the deeper parts of the

lateral walls of the main lumen and in the secondary branches, and are as a rule in a state of activity.

Frequently the lymph follicles lie partly in the submucosa and partly in the mucosa, in which case the muscularis mucosae is not present at those points. Where the glands break through the muscularis, two conditions are found. In the one case there is a clean break with the narrow muscle band cut off abruptly around the neck of the gland. In the second case the strands of muscle bend into the follicle and run parallel to the side of the gland. There may be considerable thickening of the muscle at the angle where it is bent, but the layer of muscle surrounding the gland is always thin and is usually broken below the blind end of the primary crypt and in the neighborhood of some of the secondary outgrowths. This last is similar to the arrangement of the muscularis in the duck-mole as described by Klaatsch ('93). The lymphocytes contained in these breaks in the muscle closely surround the outer surface of the epithelium of the glands.

The structure of the glands is similar in swine and in calves and in both species they show great variety of form, except in the solitary follicles of the colon of swine, where the glands are as a rule more symmetrical. As already said, the ducts of the glands in the ileocecal valve are approximately equal in diameter to the lumen of the gland itself. The latter is ordinarily perpendicular to the surface of the valve, while the lateral branches are usually short and are in some cases absent (Figs. 1, 2). Frequently small pits with few branches are seen in the valve, some of which do not extend even to the depth of the muscularis mucosae. The lymphoid tissue in the patch just below the valve is made up of contiguous follicles lying at the level of and below the muscularis mucosae, but not extending to the depth of the circular muscle. The glands in this situation may be described as intermediate in form between the pits on the valve and the more uniformly flask-shaped glands of the colon (Figs. 3, 4). Their ducts are not as wide as those of the former and are often funnel-shaped with only the widest part of the funnel containing a plug of debris (Fig. 5). They vary greatly in size and the large glands are so much branched and bent that the primary crypt may not be readily distinguished from the secondary branches, and both frequently have slender lumina. In the aggregated follicles of the colon in calves the lymph

follicles are not so crowded as in the patch immediately below the valve and are found at all levels in the submucosa. The glands are large and much branched, and neighboring glands frequently overlies one another so that the whole area appears to be undermined with channels lined by epithelium (Fig. 6). The primary crypts or main lumina of the glands are, as a rule, considerably larger than the secondary branches, and in many cases have been found empty, while the branches often contain quantities of mucus. In some instances two glands have been found opening into the common duct. In young swine each solitary follicle in the colon contains a more or less symmetrical gland with a flask-shaped lumen from which a variable number of branches are given off (Fig. 7). They open on the surface of the intestine by a comparatively slender duct, which sometimes contains a cheesy plug composed of thickened mucin and intestinal debris.

In both species, but in pigs much more frequently than in calves, many glands have been found whose lumina are more or less completely plugged. In some cases the walls of the secondary branches are still intact, while in others they appear either to be undergoing disintegration or to have been obliterated. These plugs have been mentioned by several writers and, according to Joest and Liebrecht ('10), they are composed of retained thickened mucus mixed with lymphocytes which have passed through the epithelium. Joest ('19) says that they stand on the borderline between normal and pathological, but are not to be regarded as necrotic.

The glands containing plugs always have larger lumina than those containing only mucus or mucus and a few lymphocytes, and the secondary branches may be entirely eliminated. From a study of sections through glands, in which this elimination is taking place, the first step appears to be an excessive secretion of mucus, which distends the branches of the gland and, probably through pressure, causes a breaking down of the epithelium. At the same time the lymphocytes from the surrounding follicle migrate into the gland, and the epithelium at the base of the primary crypt disappears altogether, so that in place of the gland there is an area filled with mucus, cell detritus, and fragments of nuclei, some polynuclears, some eosinophiles, red corpuscles, and invading lymphocytes (Fig. 8). As the degener-

ation advances, all trace of the secondary branches disappears and only an indefinitely staining mass of débris is left bounded by an epithelium which is almost entirely concealed by invading lymphocytes (Figs. 2 and 9). In a series of sections made through a solitary follicle from the colon of an adult pig the gland was found to have been replaced entirely by lymphocytes (Fig. 10). In the gross specimen these follicles were identical with those seen in young pigs and appeared to have the usual small orifice on the top. In sections through the glands in pigs we have frequently seen remains of vegetable matter within the gland, showing that the intestinal contents do enter these crypts. In some cases bacteria have also been found.

For purposes of comparison we have used the ceca of turkeys. In the lymphoid tissue situated just within the entrance of the ceca lie flask-shaped glands, which are identical with those found in the colon of calves and young swine. These glands were mentioned and figured by Theobald Smith ('15) when he was investigating the disease of blackhead in turkeys.

DISCUSSION AND SUMMARY.

Although the glands under discussion have been comparatively little studied, a survey of the literature shows them to be normally present in a number of birds and mammals and in man. They have been seen in the fetus of a number of the domestic animals and in the present investigation have been found in calves one day old. They probably develop as the diverticula, which have been described by Lewis and Thyng ('08) in the pig, rabbit, and man. In structure they are identical with the intestinal glands of Lieberkühn except that they extend into the deeper layers of the intestine and are much more branched. They are always surrounded by an envelope of lymphocytes, but it is not clear whether the down-growing of the gland takes place into a lymph follicle already there or whether the accumulation of the lymphocytes around the glands is a secondary step. A germ center has not been seen in any of the follicles in which glands are present.

What is the function of these glands? Their structure shows them to be invaginations of the surface mucosa, which suggests that they may be a device whereby the mucus-secreting area is greatly increased within a small compass. Further, their position at the ileocecal valve in both calves and swine and at the narrowing of the lumen of the colon in calves also suggests the function to be the providing of an excess supply of mucus where stoppage of the intestinal content is likely to occur.

According to Cornil ('73), Kelsch ('73), and Orth ('01), glands are present in the follicles of the colon of adult man. The two former found them only in cases of dysentery, but the latter found them both in diseased and in sound intestines. By Muthmann ('13) and by Theobald Smith ('15) they have been found in adult birds and by Klaatsch ('93) in the adult duck-mole. In the smaller domestic animals they are apparently more numerous in the fetus and in very young rather than in the older animals, although Retterer ('91) believed that the primary diverticula persist as crypts in the adult guinea-pig, while their secondary branches are replaced by lymphocytes. He ('92) also found later that in the horse, sheep, and cow no diverticula, extending from the follicles to the lumen of the intestine, persisted in mature animals. Our findings tend to confirm the fact of the disappearance of the glands in older animals. As has been described above, there is apparently an excessive secretion of mucus in the secondary branches followed by a breaking down of their epithelium and the replacement of the whole structure by an indefinitely staining plug, into which an invasion of lymphocytes from the surrounding follicle takes place. Sections through the follicle of an adult pig showed no trace of a gland in a solitary follicle of the colon, and Rüdinger ('91) has described a similar replacement of glands by lymphocytes in the appendix of man. Joest ('19) pointed out that the formation of the plugs is not a necrotic process and the destruction of the epithelium of the gland is probably in part at any rate mechanical, and due to the pressure exerted by the excess secretion of mucus within its branches. The multiplication of the lymphocytes in the surrounding follicle and their migration into the gland must also play an important part in its removal.

It is evident that these glands are not present in the larger domestic animals after they have reached maturity. A possible reason for their disappearance may be that they afford too ready foci for disease, and their replacement by lymph follicles is a protective action on the part of the animal system. We have not touched upon the pathology of these glands, but it has been discussed by some writers, particularly Klein, who first named them 'flask-shaped glands,' because of their appearance in the lesions of pig typhoid.

BIBLIOGRAPHY.

- CORNIL, V. 1873 Note sur l'anatomie pathologique des ulcérations intestinales dans la Dysenterie. Arch. de phys. norm. et path., Par., T. 5, p. 311.
- CZERMACK, N. 1893 Einige Ergebnisse über die Entwicklung, Zusammensetzung und Funktion der Lymphknötchen der Darmwand. Archiv. f. mikr. Anat., Bd. 42, S. 581.
- VON DAVIDOFF, M. 1887 Untersuchungen über die Beziehungen des Darmepithels zum lymphoiden Gewebe. Archiv. f. mikr. Anat., Bd. 29, S. 495.
- ELENBERGER, W., UND BAUM, H. 1915 Vergleichende Anatomie der Haustiere, Berl., 14te aufl., S. 408.
- JOEST, E. 1919 Spezielle pathologische Anatomie der Haustiere. Berl., Bd. 1, S. 440.
- JOEST, E., UND LIEBRECHT, C. 1910 Untersuchungen über die käsigen Pfröpfe am Ileumzapfen des Schweines. Monatsh. f. prakt. Tierheilk., Bd. 21, S. 322.
- KELSCH. 1873 Contribution à l'anatomie pathologique de la dysenterie aiguë. Arch. de phys. norm. et path., Par., T. 5, p. 687.
- KLAATSCH, H. 1893 Ueber die Betheiligung von Drüsenbildungen am Aufbau der Peyer'schen Plaques. Morph. Jahrb., Bd. 19, S. 548.
- KLEIN, E. 1877-78 Report on infectious pneumo-enteritis of the pig (so-called pig typhoid). Rep. Med. Off. Local Gov. Bd., Lond., vol. 7, p. 169.
- KÜCHENMEISTER, H. 1895 Beiträge zur Entwicklungsgeschichte der Darmlymphknötchen. Inaug.-Diss., 24 S., Rostock. (Cited by Oppel.)
- LEWIS, F. T., AND THYNG, F. W. 1908 The regular occurrence of intestinal diverticula in embryos of the pig, rabbit, and man. Am. Jour. Anat., vol. 7, p. 505.
- LUNGHETTI, B. 1904 Ricerche sulla tonsilla intestinale di alcuni mammiferi. Estratto dai processi verbali della R. Accademia dei Fisiocritici adunanza del 30, Gennaio.
- MARTIN, F. P. 1910 Vergleichend-histologische Untersuchungen über das Oberflächen und Drüsenepithel der Darmschleimhaut der Haussäugetiere. Inaug.-Diss., 130 S., Leipzig.
- MAY, H. 1903 Vergleichende anatomische Untersuchungen der Lymphfollikelapparate des Darmes der Haussäugetiere. Inaug.-Diss., 76 S., Giessen.

- MUTERMANN, E. 1913 Beiträge zur vergleichenden Anatomie des Blinddarmes und der lymphoiden Organe des Darmkanals bei Säugetieren und Vögeln. *Anatom. Hefte*, Bd. 48, S. 65.
- OPPEL, A. 1897 Lehrbuch der vergleichenden mikroskopischen Anatomie der Wirbeltiere, Bd. 2, S. 313.
- ORTH, J. 1901 Ueber die Beziehungen der Lieberkühn'schen Krypten zu den Lymphknötchen des Darms unter normalen und pathologischen Verhältnissen. *Verhandl. d. Deutsch. Path. Gesellsch.*, Bd. 3, S. 135.
- OSLER, W. 1878 On the pathology of the so-called pig-typhoid. *Vet. J. and Ann. Comp. Path.*, Lond., vol. 6, p. 385.
- RETTERER, ED. 1891 Origine et développement des plaques de Peyer chez le lapin et le cobaye. *C. R. d. S. et Memoires de la Soc. de Biol.*, ser. 9. T. 3, p. 871.
- RETTERER, ED. 1892 Du tissu angiothélial des amygdales et des plaques de Peyer. *C. R. hebdomad. des Séances et Memoires de la Soc. de biol.*, ser. 9, T. 4, p. 1.
- 1892 Origine et développement des plaques de Peyer chez les ruminants et solipèdes. *Ibid.*, ser. 9, T. 4, p. 255.
- 1895 Sur l'origine des follicules clos du tube digestif. *Verh. d. anat. Gesellsch.*, 9te Versammlung, s. 31.
- RÜCLINGER, N. 1891 Ueber die Umbildung der Lieberkühnschen Drüsen durch die Solitärfollikel im Wurmfortsatz des Menschen. *Sitzungsab. d. Math.-phys. Cl. d. k.-bayer. Akad. d. Wissensch. zu München*, Bd. 21, S. 121.
- SCHUMANN, P. 1907 Beiträge zur vergleichenden Histologie des Enddarmes und des Ueberganges des Mitteldarmes in den Enddarm der Haussäugetiere. *Inaug.-Diss.*, 84 S., Zurich.
- SMITH, THEOBALD 1910 Intestinal amebiasis in the domestic pig. *J. Med. Res.*, vol. 23, p. 423.
- 1915 Further investigations into the etiology of the protozoan disease of turkeys known as blackhead, enterohepatitis, typhlitis, etc. *Ibid.*, vol. 33, p. 243.
- STÖHR, P. 1889 Ueber die Lymphknötchen des Darmes. *Archiv. f. mikr. Anat.*, Bd. 33, S. 255.
- TOMARKIN, E. 1893 Lieberkühn'sche Krypten und ihre Beziehungen zu den Follikeln beim Meerschweinchen. *Anat. Anz.*, Bd. 8, S. 202.
- VOIGT, J. 1899 Beitrag zur Entwicklung der Darmschleimhaut. *Anat. Hefte*, Bd. 12, S. 51.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Swine 155. Section through a plugged flask-shaped gland in the ileocecal valve. $\times 40$.

FIG. 2. Calf 669. Section through a plugged flask-shaped gland in the ileocecal valve. $\times 40$.

PLATE 2.

FIG. 3. Swine 264. Section through a gland in the lymphoid tissue just below the ileocecal valve, to show the wide orifice. $\times 40$.

FIG. 4. Swine 264. Section through another gland in the same region, to show a plug in the primary crypt and numerous goblet cells in the branches. $\times 40$.

PLATE 3.

FIG. 5. Calf 495. Section through a gland in the lymphoid tissue just below the ileocecal valve, to show the funnel-shaped opening containing a small plug of debris. $\times 40$.

FIG. 6. Calf 450. Section through the lymphoid tissue lying just beyond the second bend in the colon to show the much branched glands. $\times 40$.

PLATE 4.

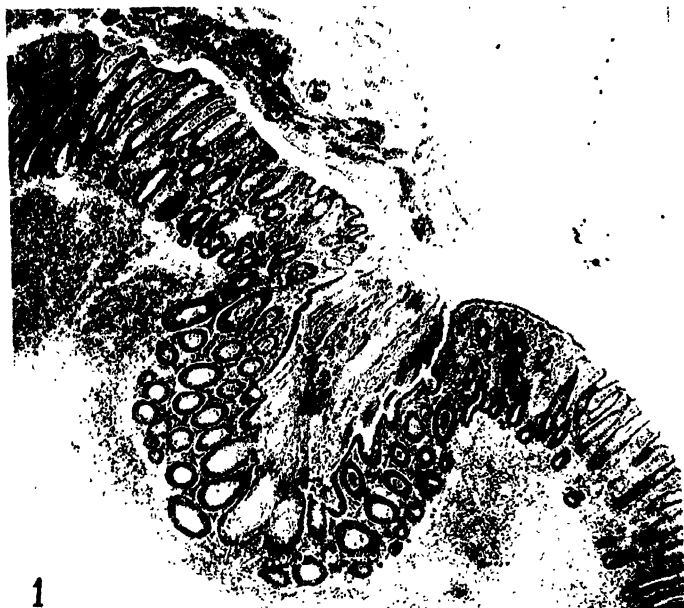
FIG. 7. Swine 260. Section through a flask-shaped gland in a solitary follicle of the colon. $\times 40$.

FIG. 8. Swine 301. Section through a gland in the lymphoid tissue lying just below the ileocecal valve, to show disintegration of the secondary branches. $\times 40$.

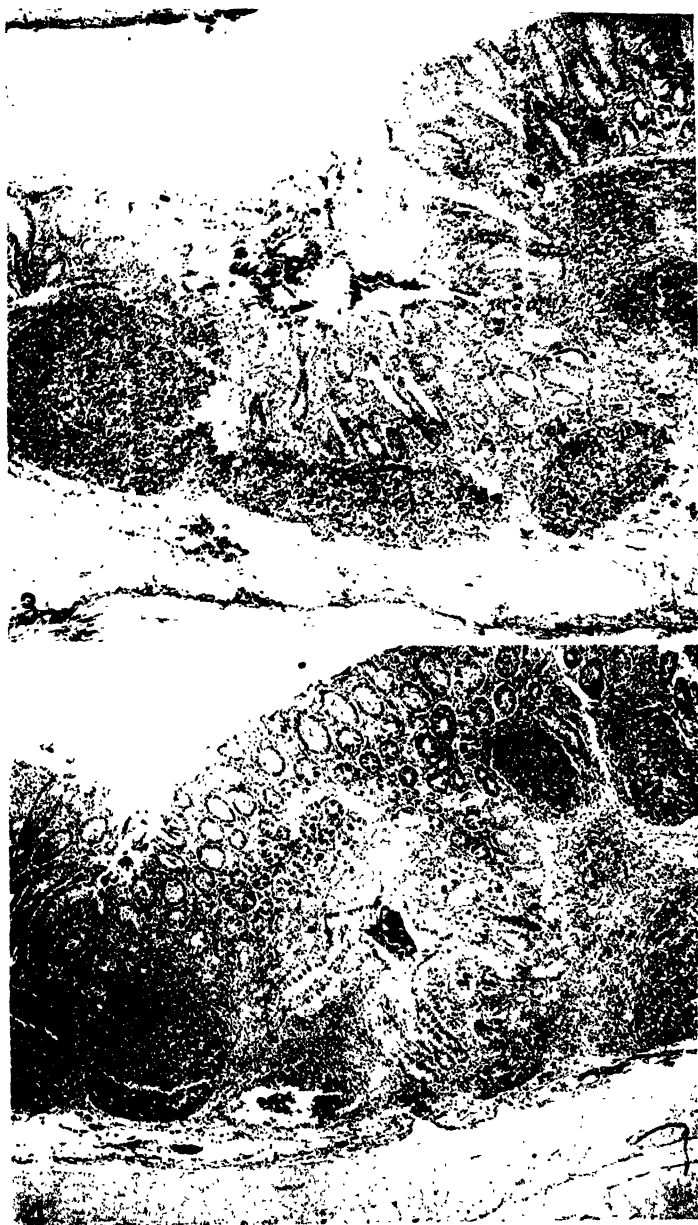
PLATE 5.

FIG. 9. Calf 700. Section through the aggregated follicles in the lower ileum, to show plugged glands. $\times 40$.

FIG. 10. Swine 271. Section through a solitary follicle in the colon, to show the replacement of a gland by lymphocytes. $\times 40$.



(Florence: Flask-shaped intestinal glands.)



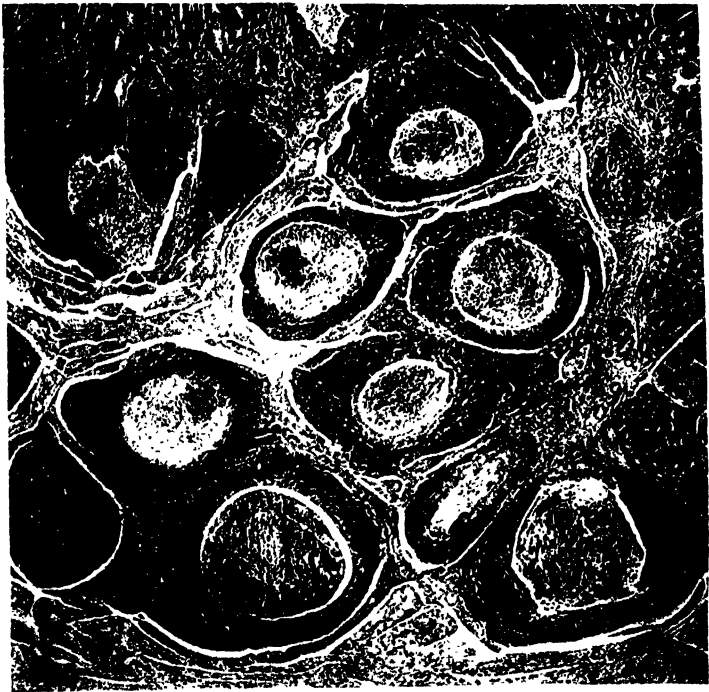
(Florence: Flask-shaped intestinal glands.)



(Florence: Flask-shaped intestinal glands.)



(Florence: Flask-shaped intestinal glands.)



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(Florence: Flask-shaped intestinal glands.)

STUDIES ON THE DISEASE OF GUINEA PIGS DUE TO BACILLUS ABORTUS.

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(Received for publication, July 7, 1922.)

HISTORY.

That a characteristic disease of guinea pigs could be produced by inoculation with cultures of *Bacillus abortus* was first pointed out by Smith and Fabyan (1912). A more detailed study of the disease was made by Fabyan (1912). Since that time the disease has become well known to workers in animal pathology, a great many of whom have utilized the susceptibility of the guinea pig to infection with this organism as a means of detecting it in animal tissues and discharges.

The observations on this disease which are recorded in this paper are largely on the pathogenicity of the organism and the resistance of the animal to infection. The data were collected preliminary to, and in connection with an experiment to determine the value of heat-killed cultures of the organism in producing an active immunity to *Bacillus abortus*, the results of which are reported in one of the accompanying papers (Hagan, 1922).

The Minimum Infective Dose.

Since it is a well recognized fact that most immunities are but relative and may be overwhelmed by a large amount of infection, though perhaps functioning perfectly for a reasonable amount, an attempt was made, in connection with the immunization experiment just mentioned, to determine the minimum amount of culture needed to produce infection regularly.

Search of the literature failed to show any data on this point. Smith and Fabyan had used comparatively large amounts of material in their work. Smil-

lie (1918) had found, in one experiment, that a standard suspension, which he had been using in his work, could be diluted at least 64 times without altering appreciably the disease process in his guinea pigs. Further than this he had not gone, but it was evident that he had not nearly approached the minimum.

The strain of *B. abortus* used in this work was isolated from the placenta of a cow (No. 898) by passage through a guinea pig. The culture had subsequently been passed through a second guinea pig immediately before use. A plain veal infusion agar slant was inoculated with a culture from the spleen of the latter animal, the tube sealed, and incubated for 48 hours at 37°C. The resultant growth was washed from the surface of the slant with a small amount of physiological salt solution, making a uniform suspension. After a little shaking this was diluted with more salt solution to make a standard density which was determined by the method of Gates (1920). A reading of 2.4 cm. was selected arbitrarily as an end-point. Such a suspension is somewhat denser than a 24 hour typhoid culture in bouillon. Hereafter in this paper such a suspension will be referred to as a standard.

From this suspension, dilutions were made by transferring 1 cc. portions from one tube to the next in series, each tube containing 9 cc. of salt solution. Thus a series of dilutions was made as follows: 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000, and 1:100,000,000. Intermediate dilutions of 1:5,000,000 and 1:50,000,000 were also made.

Preliminary work had shown that 1:100,000 dilutions of such suspensions were capable of infecting, and consequently higher dilutions were used in this series. Five guinea pigs, males, varying in weight from 355 to 410 gm. and of approximately the same age (Group V) were injected intraperitoneally with 1 cc. portions of the last five dilutions respectively. Infections resulted in the animals receiving dilutions of 1:1,000,000, 1:10,000,000, and 1:100,000,000; while the 1:5,000,000 and 1:50,000,000 failed to infect. Infection was determined by testing for the development of agglutinins and eventually, by killing the animals, observing lesions, and recovering *Bacillus abortus* culturally.

Infection by so minute a quantity of material as a 1:100,000,000 dilution of a standard suspension was a surprise. For confirmation, a second series of animals was tested with the same dilutions as before. Five male animals weighing from 415 to 470 gm. (Group V₂) received a 24 hour culture of the same strain as before. Just previous to use the strain had been recovered from another guinea pig at autopsy. In this case, infection of all animals occurred.

It was now evident that still higher dilutions of the suspension were necessary in order to approach the minimum infective dose. A third series of animals (Group V₃), consisting of eight animals weighing between 385 and 495 gm., was selected. The culture was of the same strain as before but had just been recovered from the heart's blood of a white mouse which had died within 24 hours after being inoculated with the strain. Before inoculation into the mouse the strain had been recovered from the spleen of a guinea pig which had been affected with a very severe form of the disease. The standard suspension was made up as before from a 24 hour culture, and from this in turn dilutions to 1:100,000,000, 1:200,000,000, 1:500,000,000, and 1:1,000,000,000. Two animals were injected with each of these dilutions. All became infected.

For the determination of the number of *Bacillus abortus* in the suspensions used for inoculation, the plating method described in one of the accompanying papers (Hagan, 1922) was used.

The degree of accuracy of this method is not known, as it was difficult to determine because of the small size of the organism. When several plates are made from one suspension, however, the discrepancy in the bacterial count of the various plates generally does not exceed 30 or 40 per cent, and frequently there is less than 10 per cent difference. It is advisable to add a drop of sterile blood to the surface of the agar plate at the time the suspension is added, so that the two will mix while spreading. The viscosity of the blood aids in making the film uniform over the surface, and somewhat higher counts result from the enrichment of the medium.

Although duplicate counts on a single suspension were reasonably consistent, it was found difficult to make up two suspensions which would give approximately the same count when using the Gates disappearing loop as a gauge. Thus four suspensions, standardized carefully with the Gates apparatus and plated in the same way, though at different times, gave the following counts: (a) 592, (b) 423, (c) 1,030, and (d) 1,451.

These figures represent the average counts of from two to four plates containing 0.1 cc. of a 1:1,000,000 dilution of the respective standards. The variation, amounting to over 300 per cent in the case of the extremes, must be due, in part at least, to inaccuracies in the method or operation of the opacity gauge by which the suspensions were standardized, although there is a large source of error in the pipetting when making up the dilutions. However, in estimating the number of bacteria injected into the guinea pigs, these discrepancies of count become of little consequence because of the great dilutions worked with.

If the counts given above may be accepted as representative of the true count of the living organisms in the suspensions, their average, which amounts to 874, should represent a mean which is within 100 per cent, one way or the other, of the true count in other suspensions made up in the same manner. In other words, a suspension of *B. abortus* made up in salt solution from the surface of a 24 hour slant agar culture, and having a reading of 2.4 cm. on the Gates gauge, has a mean calculated count of living organisms amounting to 8.74 billions per cc. with a minimum of about 4.5 billions and a maximum of about 13.0 billions. Since the guinea pigs were inoculated with dilutions running into the millions, these variations are reduced immediately to a few hundred organisms, at the most.

TABLE I.

Guinea pig No.	Dilution of standard suspension.	Computed No. of organisms injected.	Result.
Group V.			
2059	1:1,000,000	8,740	+
2060	1:5,000,000	1,750	-
2058	1:10,000,000	874	+
2056	1:50,000,000	174	-
2055	1:100,000,000	87	+
Group V ₁ .			
2172	1:1,000,000	8,740	+
2176	1:5,000,000	1,750	+
2179	1:10,000,000	874	+
2177	1:50,000,000	174	+
2175	1:100,000,000	87	+
Group V ₂ .			
2232	1:100,000,000	87	+
2233	1:100,000,000	87	+
2230	1:200,000,000	44	+
2231	1:200,000,000	44	+
2228	1:500,000,000	18	+
2229	1:500,000,000	18	+
2227	1:1,000,000,000	9	+
2226	1:1,000,000,000	9	+

The minimum infective dose of *Bacillus abortus* for guinea pigs has not been determined, since infection was produced in the majority of the animals with the smallest amounts of material used. The computed number of bacteria used in the experiments is given in Table I.

Many workers who have been using guinea pig inoculations for diagnostic purposes have found it possible thus to detect, at times, *Bacillus abortus*, in material from which cultures had failed, the presumption being that sparsity of the organisms accounted for the differences in results. Grounds for believing in a rather high infectivity of the organism for the guinea pig have existed, therefore. This belief is amply substantiated by the data presented above. As a means of detecting *Bacillus abortus*, guinea pig inoculations are highly efficient.

Individual Variations in Susceptibility.

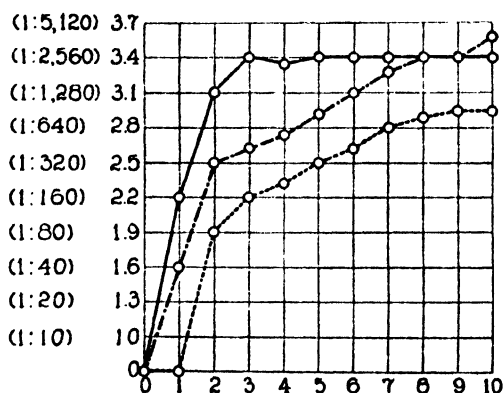
The failure of Guinea Pigs 2060 and 2056 (Table I, Group V) to become infected, although given from two to twenty times as much material as was needed to infect other animals, indicates a considerable degree of immunity in these animals. The resistance of No. 2056 was not tested subsequently, but No. 2060 was given a second inoculation at the time, and with the same material with which Group V₁ was infected (Table I). The dosage in this case was 1 cc. of a 1:1,000,000 dilution, and although this was from 100 to 1,000 times as great as that used in the animals of Group V₁, the response to the inoculation was much more tardy than that of any of the animals of the group mentioned. Although No. 2060 proved to be the most refractory of many animals tested, infection was accomplished by the second inoculation, which was of less than 10,000 organisms. Smillie reports two cases in which cultures were positive in certain parts of the alimentary tract of a bovine fetus, whereas the guinea pig inoculations from the same material gave negative results. While it is always possible that unequal distribution of the bacilli may produce such results, it is much more probable that resistant animals such as the two guinea pigs mentioned were used. The resistance would be effective only if very few organisms were present in the material injected.

The Effect of Variations in the Dosage on the Character of the Disease.

To determine the effects of dosage on the character of the resulting disease, a series of animals was inoculated, one-third with a heavy dose, another with a medium, and the other with a very small dose.

TABLE II.

Guinea pig No.	Inoculating dose.	Killed.	Spleen weight.	Spleen.	
				Organisms per gm.	Organisms per spleen.
		wks.	gm.		
2160	Undiluted.	1	2.18	269,700,000	587,946,000
2164	1:10,000	1	0.67	51,000	34,000
2165	1:1,000,000	1	0.63	450	280
2158	Undiluted.	4	1.02	2,600,000	2,650,000
2161	1:10,000	4	0.65	25,000	16,000
2166	1:1,000,000	4	0.69	106,000	73,000
2167	Undiluted.	6	1.39	24,000	34,000
2163	1:10,000	6	2.4	?	?
2157	1:1,000,000	6	1.67	39,000	65,000
2159	Undiluted.	9	3.02	48,000	145,000
2162	1:10,000	9	3.9	25,000	100,000
2168	1:1,000,000	9	1.13	1,400	2,500



TEXT-FIG. 1. Composite agglutinin curves of animals receiving different doses of *B. abortus*.

—○— Guinea Pigs 2158, 2160, 2167, and 2159. Standard suspension, undiluted.

- - - - -○- Guinea Pigs 2161, 2163, 2164, and 2162. Dilutions of standard suspension, 1:10,000.

.....○ Guinea Pigs 2157, 2165, 2166, and 2168. Dilutions of standard suspension, 1:1,000,000.

Abscissæ represent weeks, ordinates logarithms of the dilutions. Blood dilutions are in parenthesis.

A standard suspension was used for the first, a 1:10,000 dilution of this for the second, and a 1:1,000,000 dilution for the third. Four guinea pigs were inoculated intraperitoneally with each of the suspensions. All animals were bled weekly and from each group one was killed at intervals of 1, 4, 6, and 9 weeks after the inoculation. The principal results are given in Table II and Text-fig. 1.

The principal effect of changing the amount of the infecting dose is to change the time relations. A very heavy inoculation calls forth early agglutinin production and early lesions, smaller doses produce nearly the same effect but at later periods; and the smaller the dose, the longer is this period. The factor of individual variations in resistance enters rather prominently into such data as those of Table II.

The Course of the Disease.

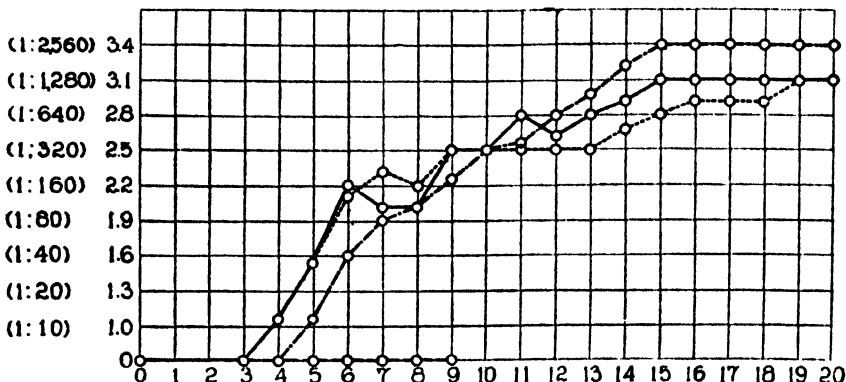
It was stated by Fabyan that the disease in guinea pigs produced by *Bacillus abortus* tended toward final recovery, although many animals might die from exhaustion and complications. These conclusions agree well with my own observations. In both his and my experiments the organism studied was isolated from cattle. Whether this is also true when the organism is isolated from other animals is a question upon which I have no data.

Inspection of all of the agglutinin curves in this and accompanying papers, except Text-fig. 2 of the present one, shows that a fall in the titer begins about the 10th week of the infection.

Two groups aggregating ten animals, the data on which are incomplete, were kept for a period exceeding 6 months after inoculation with a moderate dose of living culture of *B. abortus*. In all cases, the titer was at its height at the time of bleeding at the end of the 11th week. At the next bleeding, during the 16th week, the titer had fallen in all but one animal from the height previously occupied. During the 19th week all titers had fallen perceptibly, and at subsequent bleedings up to the time of death they were found to be falling still, slowly but definitely. When the animals were killed, after more than 6 months, only a few colonies of *B. abortus* developed in cultures from the spleen and in most cases the other organs gave no growth. It is apparent that the organisms were gradually being killed off. No animals have been kept much longer than 6 months, so that the ultimate termination of the disease is not known.

The agglutinin curves in Text-fig. 2 are exceptions to the general rule because the amount of culture used to infect was so very near the

minimum infective dose that the resultant disease was slow of onset and slow of progress. Whereas, at about the 10th week with a moderate dosage the disease would have reached its climax, in this case with a very small dose the disease was still actively progressive.



TEXT-FIG. 2. Agglutination curves of Group V guinea pigs.

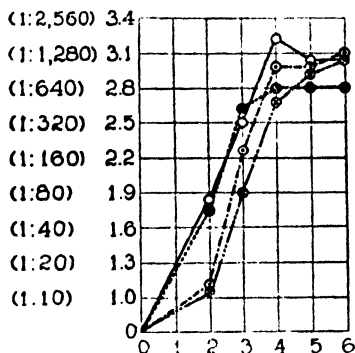
- Guinea Pig 2059.
- - -○- - - Guinea Pig 2058.
-○..... Guinea Pig 2055.
- · - · -○- Guinea Pigs 2056 and 2060.

The Alteration of Culture Virulence.

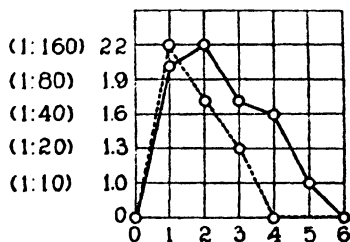
All of the work reported in this paper was done with a single strain of *Bacillus abortus* isolated less than 6 months previously. A comparison of Text-figs. 2 and 3, however, will show that enormous differences in virulence existed at different times. This difference in virulence is not simply attenuation due to *in vitro* cultivation, for Text-fig. 2, in which the virulence seems to be low, illustrates the effects of a younger cultural generation of the organism than does Text-fig. 3. This variation in virulence at different times was always encountered when dealing with small doses, so that the results in one series of animals could never be exactly duplicated in another series. It was clearly due to the influence of the bodies of the guinea pigs through which the culture had been passed.

For instance, the group represented in Text-fig. 2 was inoculated with a culture which had just been passed through Guinea Pig 1958. This animal had been

inoculated 8 weeks previous to sacrifice with a 1:1,000 dilution of a standard suspension. Only moderately severe lesions had developed, showing that the animal had been quite resistant to the infecting organism. Text-fig. 3 represents the findings in a group of animals which had been inoculated with smaller doses than those of Text-fig. 2; but the culture had just been recovered from the heart's blood of a mouse which had died within 24 hours after infection. Tracing one generation further back, it is found that the mouse was inoculated with the strain immediately after that had been recovered from the spleen of a guinea pig killed 1 week after inoculation with a large dose of culture, and that this animal had



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Agglutination curves of Group V₃ guinea pigs.

- Guinea Pigs 2233 and 2232.
- Guinea Pigs 2230 and 2231.
- Guinea Pigs 2228 and 2229.
- Guinea Pigs 2226 and 2229.

TEXT-FIG. 4. Agglutination curves of guinea pigs receiving a single injection of killed *B. abortus* culture.

- Guinea Pig 2218.
- Guinea Pig 2051.

exhibited marked lesions, especially great splenic enlargement. The organism thus had been passed through two animals immediately before use in the series. This treatment had apparently exalted the virulence to a very considerable degree. A third group of animals serves to demonstrate the same point. This group (Table I, Group V₂) was inoculated with the same amounts of suspension as in Group V. The agglutination curve, however, instead of resembling that of Group V (Text-fig. 2), was very steep, resembling that for Group V₃ (Text-fig. 3). In this case the culture had just been recovered from a guinea pig which had been inoculated with a large dose of *B. abortus* and killed in 1 week.

The Agglutinin Production of Bacillus abortus for Guinea Pigs.

Normal guinea pig serum will cause clumping of suspensions of *Bacillus abortus* only when in very low dilutions. Clumping in a dilution as high as 1:10 may be considered as evidence of artificial stimulation. This is easily accomplished by the inoculation of either living or dead organisms, but the former are much the more efficient.

Text-fig. 4 represents the agglutinin response of two normal guinea pigs to heavy doses of killed *B. abortus* suspension injected intraperitoneally. Comparison with Text-fig. 1 shows that the initial response is approximately the same as with heavy doses of living suspensions. After the 1st week, however, the curve falls, in the case of the dead culture, and within a few weeks has reached normal. If additional injections of dead culture are given, the response is slight and a titer of about 1:640 appears to be near the limit of their stimulation capabilities.

The administration of living cultures in quantities great enough to infect causes the agglutinin titer to rise to a height of 1:1,280 to 1:5,120, depending on the individual. Partial reactions in a dilution of 1:10,240 have been obtained in the case of a few individuals but this is quite rare.

Individual variation in the form of the agglutinin curves is very great, so that it cannot be predicted accurately. In general, however, the larger the initial dose the steeper is the first part of the curve. The ultimate height reached is the same, irrespective of the dose used.

Permeability of the guinea pig placenta to *Bacillus abortus* antibodies was demonstrated in two cases by the detection of agglutinins in the blood of fetuses taken from the uteri of infected mothers, the tissues of the fetus proving sterile upon adequate culture. In one case the titer of the mother was 1:1,280 and that of her fetus 1:160; in the other, that of the mother was 1:2,560, and that of the fetus 1:80. The fetal fluids were negative in a dilution of 1:20 in each case.

Natural Infection of Guinea Pigs with Bacillus abortus.

In the course of this work many guinea pigs, some normal and some infected, were kept together in large pens, and not a single instance of natural infection of the normal animals occurred. The animals were always of the same sex, as the two sexes were kept separate to prevent breeding during the experiment. A number of infected females left over from another experiment were divided into

two groups of five animals each and each group penned with a normal male. After 4 months, one group was killed. Four of the females were pregnant and the male was healthy. In the other group, agglutination tests at the end of the 3rd month indicated developing infection in the male. The group was sacrificed at the end of the 4th month and the following facts noted. There were no gross lesions to indicate the disease in the male. Cultures from the spleen yielded a few colonies, and those from the right epididymis showed heavy growths of *Bacillus abortus*. Cultures from other organs gave no growth. The agglutinin titer at death was 1:320. Three of the females were pregnant. Of the other two females, one was interesting as being the probable source of infection of the male animal. Aside from the usual lesions found in advanced cases of the disease (the animal had been inoculated about 6 months previously), there were two abscesses located in the wall of the uterus, one of which was discharging into its lumen. The left uterine horn was bound down by adhesions to surrounding tissues. The abscesses were old, thick walled, and filled with a thick, yellowish white, non-odorous pus, such as is frequently seen in epididymal abscesses.

It is not possible to say in what manner the male guinea pig became infected, but the finding of a *Bacillus abortus* abscess opening into the female generative organs and the isolation of the organism from the generative organs of the male, which animal showed little evidence of the usual systemic disease, forms a formidable chain of circumstantial evidence. Infection by ingestion is improbable in the light of past experience, and is rendered more so in this particular case by the fact that normal females had been associated for months with this same group of animals without contracting infection.

The Susceptibility of Guinea Pigs to Infection by Bacillus abortus through Ingestion.

The common experience that guinea pigs infected with *Bacillus abortus* may be kept with normal animals of their own sex with little or no danger of infecting the latter suggested that normal animals are probably not highly susceptible to feeding infection. This point was tested in the following experiment.

A standard suspension of *B. abortus* was made up and from this dilutions of 1:10, 1:100, and 1:1,000. With a small bulb pipette 1 cc. of each suspension was placed directly into the mouth of young normal animals. No difficulty was experienced in getting the animals to swallow the entire amount, so the exact dosage is known for each animal.

The two animals receiving the undiluted and the 1:10 dilution of the standard suspension became infected, the other two given the higher dilutions remained well. Agglutinin production in the infected animals did not become evident until the 3rd week following infection, a lag seen among inoculated animals only in those receiving a very minute dose.

It appears from this experiment that the susceptibility of guinea pigs to feeding infection with *Bacillus abortus* is actually quite low, as had been surmised. It is computed that the ratio between the minimum infecting dose of *Bacillus abortus* by inoculation and by feeding is at least as great as 1 to 100 million.

SUMMARY AND CONCLUSIONS.

1. The infectivity of *Bacillus abortus* for guinea pigs is extremely great. It is computed that somewhat less than 100 organisms are required to infect most animals.

2. Occasional animals are encountered having much more resistance to *Bacillus abortus* infection than is possessed by the average. The most resistant animal encountered was infected by a number of organisms computed to be less than 10,000.

3. The principal effect on the character of the *Bacillus abortus* disease, caused by varying the size of the infecting dose, is a change in the time relations. Very small doses produce an infection of slower course than do larger doses, but the eventual results are practically the same.

4. Judging by the agglutination curves, guinea pigs inoculated with large or medium sized doses of *Bacillus abortus* generally begin to gain mastery over the disease about the 10th week after inoculation. At this time the agglutination curve begins to decline. As was first shown by Smillie, the numbers of bacteria in the body organs begin to decline at about the 4th week but the change is rather slow until about the 10th or 12th week when a rapid decline begins, coincidently with the fall in the agglutinin titer. When the inoculating dose is small

the course of the disease may be much prolonged. The defensive forces of the body would appear to be quite inactive until a certain stage in the disease is reached.

5. The virulence of *Bacillus abortus* cultures for guinea pigs may be raised or lowered by appropriate passage through these animals.

6. Normal guinea pig blood will not agglutinate *Bacillus abortus* in a dilution of 1:10. Immune agglutinins are easily produced by inoculations with dead or living organisms. Agglutinins for *Bacillus abortus* are capable of passing the placental filter.

7. There appears to be little or no danger of normal guinea pigs becoming infected by association with diseased animals of their own sex. One instance is reported, however, of the infection of a male animal that had cohabited with infected females.

8. The susceptibility of guinea pigs to infection through ingestion of *Bacillus abortus* is relatively slight.

BIBLIOGRAPHY.

- Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Hagan, W. A., *J. Exp. Med.*, 1922, xxxvi, 711.
Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.
Smith, T., and Fabyan, M., *Centr. Bakt., 1te Abt., Orig.*, 1911-12, lxi, 549.

THE VALUE OF HEAT-KILLED CULTURES FOR THE PREVENTION OF THE BACILLUS ABORTUS INOCULATION DISEASE OF GUINEA PIGS.

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(Received for publication, July 7, 1922.)

The great economic importance of infectious abortion in cattle and swine, particularly in cattle, has provided the stimulus for many experiments aimed at the prevention of the disease through the use of preparations made from the *Bacillus abortus* and its products. Naturally the use of killed cultures of the organism was among the first of these procedures attempted and it has, perhaps, been the method most extensively tried. In spite of this the exact immunizing value of the killed cultures is not known though it is quite generally thought at the present time to be slight. The interpretation of results of immunization experiments against infectious abortion in cattle is attended with certain inherent difficulties which have left nearly all such experiments open to question.

The disease of guinea pigs due to inoculation with *Bacillus abortus*, first described by Smith and Fabyan (1912), seemed to offer a more favorable opportunity for studying the mechanism of the process of immunity than the cattle disease, since it is not attended with the difficulties found in the latter. Such a study was taken up, therefore, not only for the light which might be cast by it on the general biology of the organism but for the possible assistance which it might render toward solving the cattle problem. It was realized, of course, that results obtained in working with one species of animal cannot safely be applied to another species, by inference, unless there is strong corroboratory evidence.

HISTORICAL.

Previous attempts to immunize guinea pigs to *B. abortus* by the use of killed cultures have been made by Ascoli (1915), and by Stafseth (1920). Stafseth found that no immunity was produced by the procedure, but his work is of little value because the size of his infecting dose was so enormous as to overwhelm any but the strongest of immunities.

Ascoli's work may be criticized similarly, although it is evident that he appreciated the importance of a reasonably small infecting dose and made an effort to attain it. His immunizing cultures were killed with ether. Both single large doses of the immunizing suspension and small repeated doses were tried. The infecting doses varied from one series of animals to another, but the smallest used was 1:100,000 of an agar slant growth. The animals were killed after periods of time varying from a few days to several months. The agglutinin titer of the blood was determined when the animals were killed, and cultures were made from the spleen. The nature and extent of the lesions apparently were not considered.

The animals previously subjected to the immunization process as well as normal animals used as controls developed agglutinins for *B. abortus* and the organism was recovered from the spleens of all. It was concluded, therefore, that the use of killed cultures of *B. abortus* was ineffectual in preventing subsequent infection by inoculation.

Plan of Experiment.

Since previous work, supported by similar observations dealing with the disease in cattle, indicated that the degree of immunity produced in the animals probably would be slight, if not nil, it was planned to carry on observations upon a quantitative basis in an effort to detect immune processes which might not be of sufficient degree to afford complete protection.

A group of twenty-four female animals was selected from the stock, uniformity of age and size being sought. All were young, half grown animals weighing in the neighborhood of 350 gm. Agglutination tests with *Bacillus abortus* antigen showed the blood of all to be negative in a dilution of 1:10. One-half of the animals (twelve) were subjected to preliminary injections of killed cultures and finally all, with the exception of two animals from each group which were preserved as controls, were inoculated with living culture. Throughout the experiment all animals were kept together in a single large pen.

Evidences of immunity in the group of animals receiving the pre-

inary treatment were sought by comparing the group with the untreated in the following respects: (a) changes in body weight; (b) differences in the agglutinin curves; (c) extent and character of the lesions, with especial reference to the size and condition of the spleen; and (d) number of *Bacillus abortus* cultivable from the spleen.

Immunization.

The culture used throughout this work was a typical strain of *B. abortus* isolated from a bovine placenta, Case A 898, by passage through Guinea Pig 1865. It grew readily on plain veal infusion agar, provided the cultures were hermetically sealed. For antigenic material this strain was grown 48 hours on plain agar in a Blake flask which had been sealed with wax. A suspension of the resulting growth was made in physiological salt solution and heated at 60°C. for 1 hour. Both cultural and animal inoculation tests proved the suspension to have been sterilized. It was diluted to a reading of 2.4 cm. on the gauge devised by Gates (1920) and stored, without preservation, in the refrigerator until ready for use.

The twelve animals selected for immunization were given intra-peritoneal injections of 1 cc. of the killed bacterial suspension weekly. The manner in which the animals were reacting to the injections was gauged by the agglutination curve. The weekly injection of rather large doses of killed bacterial suspension appeared to exert little effect on the health of the animals, although reference to Table I will show that the group did not gain in weight so well as did the untreated group. The gain in weight during the period of immunization for the treated group averaged 162 gm., while for the other group the average was 231 gm.

The curve in Text-fig. 1 is typical of the agglutinin response in all of the treated animals. After a sudden rise following the first injection there was a slow rise until an average titer of 1:640 was reached, beyond which point it appears that there is no stimulation by the use of dead cultures. After six weekly injections the process was stopped and some time was allowed to elapse before the test inoculations were made, in order to allow the body to recover its equilibrium which it was thought might have been upset by the stress of the immunizing process. At the end of the 3rd week following the last dose of killed culture, the agglutinin titers began to show evidence of falling and the time was considered ripe for inoculation with the living culture.

The Infecting Dose.

The strain of *Bacillus abortus* which had been used in the preparation of the immunizing suspension was also used for infection.

The culture had been passed through Guinea Pig 2106 just prior to use. The growth from a 24 hour sealed slant agar culture was washed off with physiological salt solution and the resulting suspension diluted to make a reading of $\frac{1}{4}$ cm. on the Gates loop gauge. This makes a suspension somewhat denser than a 24 hour typhoid culture in bouillon. Dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were made from the original suspension. The last three dilutions were used for inoculating, 1 cc. being injected intraperitoneally in each case.

In the matter of dosage, it was sought, in some of the animals, to approach rather closely to the minimum amount necessary to produce infection; in others to give a moderate dose; and in a few to give what might be considered a comparatively large dose. The question of the minimum infective dosage of suspensions of *B. abortus* is discussed elsewhere (Hagan, 1922). Reference to the communication cited will show that the smallest dose administered was probably several times the amount actually needed to produce infection. The individual variation in resistance is so great that smaller doses than the minimum amount given in this experiment could not safely be depended on to infect all normal animals, although $\frac{1}{100}$ of this amount probably would have infected most guinea pigs.

The animals were killed and examined in pairs consisting of one animal from each group. Some were killed within 3 weeks after the test inoculation; others in 6, 9, and 12 weeks. All were bled regularly at weekly intervals and the agglutination curves obtained.

RESULTS.

Body Weight.—A complete weight record will be found in Table I. Analysis of this shows that during the entire experiment the average gain in body weight was approximately the same in the two groups; *i.e.*, immunized group 308 gm., non-immunized 304 gm. At the same time two animals used as controls, which were not subjected to any experimentation except periodic bleeding for agglutination work, made an average gain of 430 gm. It has already been stated that during the immunization process the treated animals were outstripped in the amount of weight gained by some 70 gm. on the average for the group. From the time of inoculation to the time of death the position was reversed, the immunized animals gaining 109.5 gm. while the non-immunized gained only 53 gm. This margin undoubtedly would have been widened if the animals had been allowed to live longer, as

is indicated by the comparatively great weight losses suffered by the non-immunized animals which were allowed to live the longest.

The Agglutination Curves.—At the time of administering the living culture, the agglutinin titer of the immunized group averaged about 1:20, while the bloods of the non-immunized were negative at a dilution of 1:10. Text-fig. 1 shows the behavior in the immunized control animals. Following the last injection of killed culture there is a gradual decline in the curve lasting some 13 weeks before it reaches the base-line. Text-figs. 2 to 4 show the curves in the inoculated animals receiving different amounts of infective material. The latter halves of these curves show that there is practically no difference in the agglutinin response to the infecting dose in the two groups, and that the size of the infecting dose has made little difference in the agglutinin response. In Text-figs. 3 and 4 the smaller doses have produced a delay in the agglutinin production, and the curve for the immunized group falls appreciably before the effect of the test inoculation becomes operative. In the case of the comparatively heavy inoculation represented by Text-fig. 2 this fall does not occur.

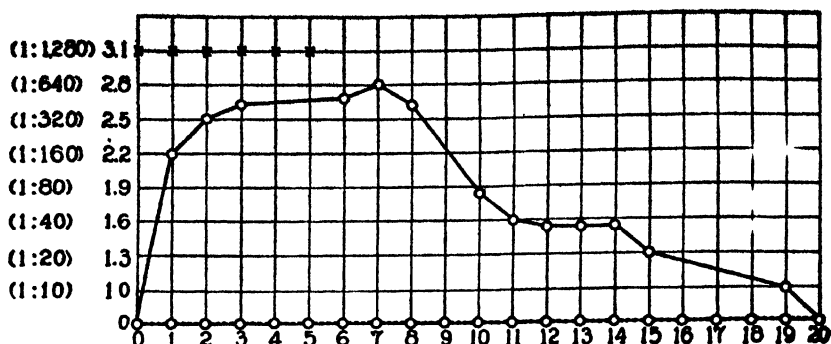
Differences in Extent and Character of Lesions.—All of the inoculated animals became infected, thus showing that the treatment was ineffectual in preventing the disease. Certain differences in the character of the disease process have been noted, however, as a result of the preliminary treatment. These have to do with the spleen, lymph glands, and kidneys.

The spleen presented the most striking lesion of the disease in all cases, immunized or non-immunized. Reference to the spleen weights in Table I shows that enlargement occurred in all cases except in Guinea Pigs 2083 and 2071, both of which were immunized animals which had received small doses of the infecting culture and were killed early. The splenic enlargement, with one exception, was greater, however, and usually much greater in the non-immunized than in the immunized group. The spleen weight for the former group averaged 4.87 gm., while for the latter it was only 3.1 gm., a difference of more than 50 per cent. There is no evidence that the character of the process in the spleen is different in the two groups. It is probable that the difference is only a matter of degree; *i.e.*, that in the immunized group the process is only delayed.

TABLE I.

Guinea pig No.	Immunised.	Infected.	Length of time lived.	Initial weight.	Maximum weight.	Weight when killed.	Loss in weight from maximum.	Change in weight during immunization.		Change in weight during disease.		Spleen weight.	Agglutinin titre when killed.	Spleen.	
								gm.	gm.	gm.	gm.			Bacteria per gm.	Bacteria per organ.
2073	+	Control.	24	410	720	720	0	+190	+100	1.07	1:40	0	0	0	0
2081	-	"	24	405	740	740	0	+215	+95	1.06	0	0	0	0	0
2074	+	10 ⁻⁴	24	390	670	670	0	+155	+90	4.12	1:5,120	?	?	?	?
2082	-	10 ⁻⁴	24	410	745	725	20	+245	+70	7.34	1:1,280	807,000	5,900,000	807,000	5,900,000
2083	+	10 ⁻⁴	24	370	690	690	0	+170	+110	1.07	1:160	83,000	88,000	83,000	88,000
2090	-	10 ⁻⁴	24	430	680	680	0	+190	+70	3.09	1:640	43,000	124,000	43,000	124,000
2071	+	10 ⁻⁴	24	335	710	710	0	+185	+130	1.19	1:320	615,000	731,000	615,000	731,000
2076	-	10 ⁻⁴	24	465	755	720	35	+250	-10	6.01	1:320	367,000	2,205,000	367,000	2,205,000
2070	+	10 ⁻⁴	42	340	645	645	0	+75	+110	3.00	1:2,560	123,000	369,000	123,000	369,000
2077	-	10 ⁻⁴	42	395	640	530	110	+200	-85	5.49	1:5,120	196,000	1,078,000	196,000	1,078,000
2067	+	10 ⁻⁴	42	370	660	630	30	+160	+100	2.48	1:1,280	198,000	491,000	198,000	491,000
2080	-	10 ⁻⁴	42	385	760	760	0	+235	+130	2.59	1:1,280	42,000	109,000	42,000	109,000
2086	+	10 ⁻⁴	63	400	750	750	0	+220	+90	2.24	1:1,280	12,500	38,000	12,500	38,000
2088	-	10 ⁻⁴	63	430	1,000	1,000	0	+240	+300	2.23	1:2,560	?	?	?	?
2075	+	10 ⁻⁴	63	410	850	850	0	+180	+240	4.8	1:2,560	33,800	162,000	33,800	162,000
2079	-	10 ⁻⁴	63	305	725	725	0	+215	+180	2.74	1:5,120	?	?	?	?

		Control.	85	415	800	800	800	0	+160	+170	1.07	0	0	0
2068	+	"	85	385	915	915	915	0	+260	+235	1.07	0	0	0
2085	-		85											
2069	+	10 ⁻⁴	85	335	560	480	480	80	+125	- 10	5.92	1:5, 120	109,000	645,000
2078	-	10 ⁻⁴	85	390	710	540	540	170	+230	-115	8.75	1:5, 120	75,500	662,000
2072	+	10 ⁻⁴	85	365	730	730	730	0	+185	+155	3.26	1:2, 560	11,300	36,900
2087	-	10 ⁻⁴	85	390	915	670	670	245	+260	- 30	4.32	1:2, 560	38,000	105,000
2084	+	10 ⁻⁴	85	330	600	570	570	30	+140	+ 80	2.97	1:2, 560	43,000	128,000
2089	-	10 ⁻⁴	85	400	830	690	690	140	+230	+ 20	6.17	1:5, 120	58,600	361,500



TEXT-FIG. 1. Composite agglutinin curves of normal control, and of immunized but non-infected animals.

—○— Composite curve of Guinea Pigs 2068 and 2073, animals which were immunized but not infected. The times of injecting the killed suspensions of *B. abortus* are indicated at the points x.

.....x Composite curve of Guinea Pigs 2081 and 2085, animals which were untreated but allowed to live throughout the experiment in the same pen with the infected animals.

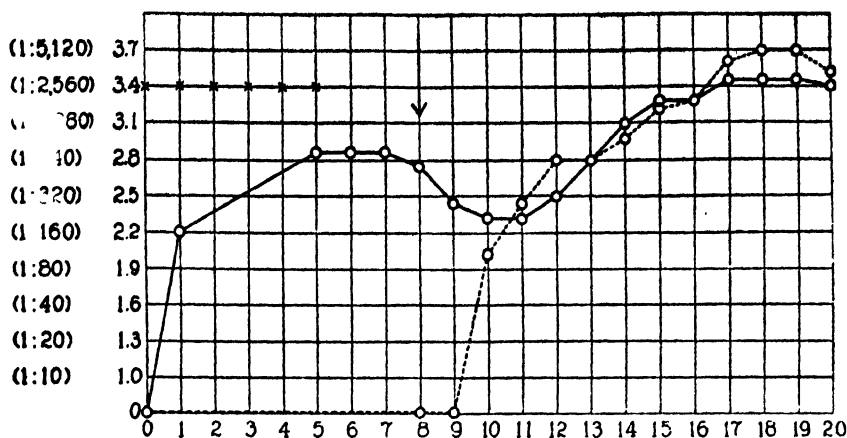
In this and all succeeding agglutinin curves, the abscissæ represent weeks, and the ordinates the logarithms of the dilution of the blood. The actual blood dilutions are given in parenthesis.



TEXT-FIG. 2. Composite agglutinin curves of immunized and non-immunized animals, infected with 1:1,000 dilution of a standard suspension of *B. abortus*. The times of injecting killed suspension of *B. abortus* are indicated by x, the time of infection by the arrow.

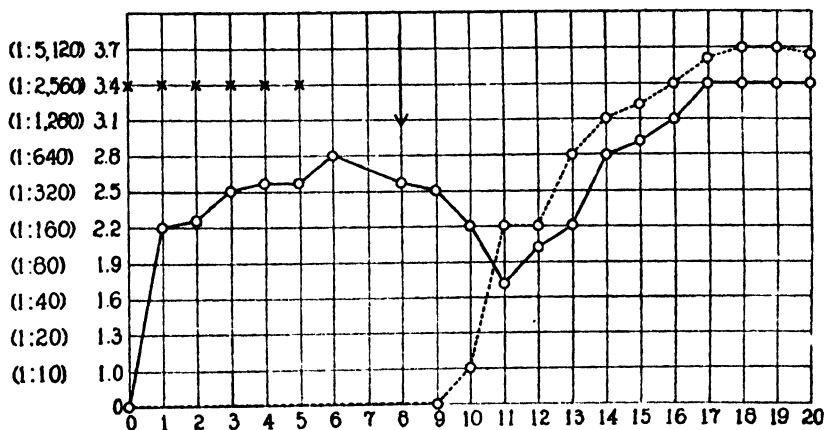
—○— Guinea Pigs 2068 and 2074, immunized.

.....x Guinea Pigs 2078 and 2082, non-immunized.



TEXT-FIG. 3. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:100,000 dilution of a standard suspension of *B. abortus*.

- Guinea Pigs 2070, 2072, 2083, and 2086, immunized.
-○..... Guinea Pigs 2077, 2087, 2088, and 2090, non-immunized.



TEXT-FIG. 4. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:1,000,000 dilution of a standard suspension of *B. abortus*.

- Guinea Pigs 2067, 2071, 2075, and 2084, immunized.
-○..... Guinea Pigs 2076, 2079, 2080, and 2089, non-immunized.

Enlargement of the lymph glands occurred, coincident with splenic enlargement, and was more pronounced in the non-immunized animals than in the immunized.

A striking lesion in the non-immunized animals which lived the longest and in several of the non-immunized animals which were killed early occurred in the kidneys. This lesion is frequently found in the later stages of the guinea pig disease and was described by Smith and Fabyan (1912). It consists in cellular infiltration in the early stages, followed later by sclerosis. That the lesion is of great import to the body economy is rendered probable by the fact that as the severity of the sclerosis increases emaciation of the body keeps pace. The condition is always accompanied by a very large spleen, but great enlargement of the spleen is sometimes found without accompanying kidney disease and emaciation. This kidney lesion was found among the immunized animals but once, in Guinea Pig 2069, one of the animals which received a heavy infecting dose.

The Number of Bacillus abortus Present in the Spleen.—The manner of determining the bacterial count of the spleen is described in the appendix. The counts are given in Table I. It will be noted that the bacterial count per gram of splenic tissue is greater in the immunized than in the other group, while the reverse is true when the bacterial content of the entire organ is considered. The explanation for this lies in the greater enlargement of the organ in the latter group. Since this enlargement is largely due to engorgement of the sinuses with blood, the bacterial concentration per unit of volume is decreased by dilution. Taking the entire organ, however, there is an appreciably greater number of cultivable *Bacillus abortus* present in the spleens of the non-immunized than in those of the immunized animals.

SUMMARY AND CONCLUSIONS.

From the data presented it seems permissible to draw the following conclusions.

1. Guinea pigs cannot be rendered immune to the *Bacillus abortus* inoculation disease by treatment with heat-killed cultures of this organism. This agrees with the work of Ascoli, and with the general conclusions of many that dead cultures confer very little or no immunity to infectious abortion in cattle.

2. The progress of the disease can be delayed appreciably by such treatment. This is supported by the following observations: (a) the loss in body weight due to the disease is delayed; (b) the development of splenic enlargement is partially inhibited or delayed; (c) the development of renal lesions is delayed or prevented; and (d) the rapidity of multiplication of *Bacillus abortus* in the splenic pulp is reduced.

APPENDIX.

Technique of Bleeding Guinea Pigs for Agglutination Tests.—The repeated bleeding of guinea pigs for periodic agglutination tests has been accomplished in an easy and fully satisfactory manner. Many animals have been bled ten to twenty times without injury and without difficulty in obtaining the requisite amount of blood for macroscopic tube tests. The method is as follows:

The laboratory should be warm so as to encourage the circulation in the peripheral vessels. One of the ears is rubbed briskly with a pledget of cotton soaked in xylol, and then dried with a clean bit of cotton; the blood vessels are examined and one of them nicked transversely with the point of a scalpel. The vessels can be seen to better advantage if some form of illumination below the ear is used. A vigorous flow of blood generally wells up from the small incision. The blood is taken into a 0.1 cc. serological pipette which is provided, for convenience, with a length of rubber tubing and a mouthpiece. One pipetteful (0.1 cc.) is immediately delivered into 0.9 cc. of citrated physiological salt solution, making a dilution of the blood of approximately 1:10. If necessary to work with low dilutions, two pipettefuls (0.2 cc.) may be delivered into 0.8 cc. of salt solution, making a dilution of 1:5. The tubes are shaken up and placed in the refrigerator over night. The next day as much as 0.7 to 0.8 cc. of a perfectly clear dilution of plasma may be pipetted off.¹ Since only 0.5 cc. is needed for the agglutination tests, the quantity is ample. The citrate-salt solution consists of 0.9 per cent sodium chloride and 2.0 per cent sodium citrate in distilled water. This amount of citrate

¹ The dilution figures in this and accompanying papers are given in terms of the whole blood. The serum or plasma dilutions may be roughly computed as twice as great as that of the whole blood.

does not have any effect on the agglutinin titer so far as could be determined by checking against sera obtained by defibrination. The citrated plasma has the advantage over most sera in that it is perfectly clear and untinged with hemoglobin.

Agglutination Technique.—The technique of carrying out the agglutination tests is practically that described by Smillie, Little, and Florence (1919).

The antigen has been freshly prepared and no preservative added, although equally good results appeared to be obtained by using an antigen preserved with 0.25 per cent formalin and kept stored in the refrigerator for several months. Antigen preserved with 0.5 per cent phenol is not satisfactory because it induces a permanent cloudiness of the suspension which renders the reading of partial reactions difficult.

The Determination of the Approximate Numbers of Bacillus abortus Present in the Spleen.

Preparation of Splenic Tissue for Plating.—The following method was used for obtaining a measured and representative sample of splenic tissue for plating purposes.

A portion of the organ representing from 0.5 to 1.5 gm. is removed aseptically to a sterile weighed potato tube and its weight determined. The fragment is then crushed thoroughly in a manner to be described and diluted with nine times its weight of physiological salt solution. After thorough mixing, 0.1 cc. portions are used for plating, unless it is suspected that the bacterial count will be greater than 100,000 organisms per gm. of tissue, in which case 0.1 cc. of a 1:5 or 1:10 dilution of the original suspension is used.

For grinding or crushing the splenic tissue efficient crushers were made from heavy walled culture tubes in the following manner.

Tubes of as nearly perfect form as possible are selected from the stock and arranged in pairs, one tube of which is of such size that it will just slip easily into the other like the plunger of a syringe. The smaller tube is then constricted in its middle part by heating in a blast flame and drawing it out. The larger tube is shortened by cutting it off at such length that its mouth will be about at the level of the middle of the constriction of the smaller tube when the latter is placed within it. The inner tube is used as a pestle while the outer serves as

a mortar. The surface of the pestle is roughened by rotating it in contact with a piece of carborundum paper held in the hand. The inside of the mortar has not been altered although it probably could be improved somewhat by etching with hydrofluoric acid. Great roughening is not to be desired since the mortar is apt to be cut and broken, in the process of grinding, by sharp edges on the pestle. For the same reason abrasives such as fine sand or carborundum powder cannot be used. The apparatus is intended for crushing purposes rather than for grinding.

The crusher is wrapped in paper and sterilized by hot air. The piece of weighed spleen is placed in the larger tube and forced to the bottom with the pestle. It is now compressed and broken up by rotating and exerting gentle pressure on the pestle, the soft pulp being forced up between the sides of the pestle and the inner wall of the mortar. If the fit between the two elements of the crusher is good, by the time the pulp reaches the space in the upper end of the mortar left by the constriction of the shaft of the pestle it will be thoroughly reduced to a homogeneous paste. When the crushing has been completed small portions of salt solution are pipetted into the mortar and the pestle slowly lifted while being rotated. The vacuum in the bottom of the pestle causes the fluid to pass the pestle, thereby cleansing it of the pasty pulp mass. Several repetitions of this process generally leave the pestle clean, after which it is removed and the remainder of the quantity of salt solution needed to make a 1:10 dilution added.

Plating Methods.—With *B. abortus* ordinary plating methods are useless because this organism requires a peculiar gaseous environment which is not satisfied by the free atmospheric exchange of the ordinary Petri dish. For obtaining suitable gaseous conditions for the cultivation of the *B. abortus* in plate culture, there have been available the methods of Nowak (1908) and of Huddleson (1920). Since both of these are somewhat involved, while furthermore simple hermetic sealing of the tubes had proven an entirely satisfactory method of obtaining growth in tube cultures, it was decided to try this method with plate cultures in lieu of the others. A simple and satisfactory method of sealing the plates has been devised and the results in obtaining growth of *B. abortus* have been entirely satisfactory.

Pieces of double strength window glass were cut into 12 cm. squares, and others into pieces 12 by 24 cm. in order to handle two cultures as a unit. The edges of the glass were ground off to remove sharp points and corners. These squares were used as bases upon which the halves of the Petri dishes containing the cultures were sealed. The steps in the method are as follows:

1. Paraffin of a melting point of 55–60°C. is placed in a shallow vessel and heated until it smokes. The high temperature serves to sterilize the paraffin. When it has cooled to 60–70°C., a few degrees above its melting point, it is ready for use.

2. A glass square is placed on the table and its upper surface thoroughly traversed with the Bunsen flame. This not only sterilizes the surface but serves to warm the glass.

3. The lower half of the Petri dish containing the inoculated agar is grasped, open surface downward, with the tips of the fingers and the edges dipped into the molten paraffin to a depth of 5 to 8 mm. The entire margin should not be submerged at one time, otherwise when the plate is lifted the rush of air under the edge is apt to cause spattering of the paraffin on the agar surface. This may be avoided easily by tilting the plate when submerging. Since the temperature of the glass of the Petri dish is lower than the melting point of the paraffin, a layer of the latter in a congealed state adheres to the plate when it is lifted.

4. The plate is placed on the center of the glass square and gently pressed down. Since the square is warm, and there has been no delay between the dipping of the edges of the plate and planting it on the glass surface, an excellent seal between the plate edge and the glass base is formed.

5. To assure a perfect seal, the flame of a micro burner is run quickly around the joint between plate and base. If done properly the surface only of the paraffin layer will be melted and all cracks and crevices will be filled. If too much heat is used the glass will become heated through and will cause the paraffin to spread out in a thin layer on the base, thus forming an imperfect seal, or at best a thin seal which is apt to rupture through when a partial vacuum has formed within the plate.

Even when hermetically sealed, poured plates are not satisfactory for quantitative work when dealing with *B. abortus* for the reason that the organism will not develop in anaerobic or semianaerobic conditions. The deep lying bacilli will, in most instances, fail to develop and those that do grow will produce colonies so minute that counting is difficult or impossible. Surface inoculation has been used exclusively in this work. 0.1 cc. of the splenic suspension is pipetted onto the surface of the agar plate and spread uniformly by means of a bent glass rod. This amount of fluid will spread evenly over the surface of the agar without leaving an excess of fluid. The plate is then tilted, in an inverted position, on the rim of the lid to allow the surface to dry somewhat before it is sealed.

Growth generally appears within 3 to 4 days, though sometimes not for 5 or 6 days. Within 24 hours of the time when colonies can first be seen, they will have reached a diameter of 1 mm. if there is not too much crowding. After this, growth is comparatively slow, but if very few colonies are present they may reach a diameter of from 3 to as much as 6 mm. The colonies have no tendency to spread or coalesce. Because of the discrete character of the growth as many as 1,000 colonies may grow on a single plate without evidence of interference with each other, other than that their ultimate size will not exceed 1 mm.

Generally all colonies appear at the same time and grow at about the same rate of speed, so that they have about the same size. In some instances, however, two crops of colonies may appear. In these cases the second crop will become visible 1 to 3 days after the first as minute colonies lying between the first. The second crop colonies always remain small, seldom reaching a diameter of 0.5 mm. even when they are not crowded and are subjected to prolonged incubation.

Because of this phenomenon, it is always advisable to incubate the plates for 3 or 4 days following the appearance of the first crop of colonies before making the colony count.

BIBLIOGRAPHY.

- Ascoli, A., *Z. Infektionskrankh. Haustiere*, 1915, xvii, 156.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Hagan, W. A., *J. Exp. Med.*, 1922, xxxvi, 697.
Huddleson, I. F., *Michigan Agric. Exp. Station, Bull. 49*, 1920.
Nowak, J., *Ann. Inst. Pasteur*, 1908, xxii, 541.
Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.
Smith, T., and Fabyan, M., *Centr. Bakt., 1te Abt., Orig.*, 1911-12, lxi, 549.
Stafseth, H. J., *Michigan Agric. Exp. Station, Bull. 49*, 1920.

THE SUSCEPTIBILITY OF MICE AND RATS TO INFECTION WITH *BACILLUS ABORTUS*.

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(Received for publication, July 7, 1922.)

It was observed by Holth (1911) that an acute disease resulted when mice and rats were inoculated intraperitoneally with cultures of *B. abortus*. It was stated that from 0.25 to 0.5 cc. of a serum bouillon culture in mice, and from 0.5 to 1 cc. in rats, was sufficient to produce a prostrating disease with fatal termination in from 2 to 5 days. The disease was essentially an intoxication. At autopsy the principal lesion found was a moist, reddened peritoneum with large numbers of the specific organism and a few leucocytes on its surface. Culturally, a few organisms could be found in the blood after death.

These observations were confirmed, in general, by Zwick and Zeller (1912), and by Ascoli (1915). In Ascoli's tables, it is noted that *B. abortus* was recovered culturally in several cases, presumably from the spleen, after periods of time as great as 2 months after inoculation, when because of insufficient dosage or artificial immunization death did not occur from the acute form of the disease. Ascoli does not mention the presence or absence of pathological changes in these cases. Zwick and Zeller noted an occasional acute swelling of the spleen, in addition to the changes described by Holth.

Fabyan (1912) showed that a type of disease entirely different from that just described might be produced in mice by intraperitoneal or subcutaneous injection of *B. abortus*. This disease was of a chronic nature, similar in many respects to that produced by the organism in guinea pigs. Although his dosage was rather high he does not mention having observed the acute disease previously described. It is possible that his statement that "many of the animals died from complications, frequently without apparent cause" refers to this form of the disease. 3 to 4 weeks after inoculation he found the spleens enlarged in thirteen of nineteen animals which had survived from a group of about thirty inoculated. Lesions in the spleen, lungs, liver, kidneys, lymph glands, and epididymis were found. Rats were inoculated also but no effect of the inoculations was detected.

INTRODUCTION.

The work just described made it clear that mice and rats could be infected experimentally with *Bacillus abortus* when a large amount of

infective material was employed for inoculation. Whether these animals could be infected with such small amounts as to make them useful in detecting the organism in diagnostic work with infectious abortion of cattle, and whether they were sufficiently susceptible to infection by ingestion to make the wild varieties a possible factor in the propagation and transmission of the disease among cattle were questions unsolved. To the working out of these two questions this paper is devoted.

The Susceptibility of White Mice to the Inoculation Disease.

Ten young, white, male mice of about the same size and age were selected from the stock and divided into five pairs, each pair being placed in a jar by themselves. The animals of the first pair were inoculated differently, one with a 48 hour serum bouillon culture of *Bacillus abortus*, and the other with an undiluted suspension of *Bacillus abortus* washed from a 48 hour agar slant culture. The second pair received a 10^{-3} , the third a 10^{-4} , the fourth a 10^{-5} , and the fifth a 10^{-6} dilution of this suspension. All were inoculated with 0.5 cc. of dilution, intraperitoneally. One guinea pig was inoculated with 0.5 cc. of the 10^{-6} dilution as a check on the virulence of the culture and for purposes of comparison.

The suspension above referred to was made by growing a bovine strain of *Bacillus abortus*, recently recovered from a lymph gland of Guinea Pig 2082, in a sealed tube of plain veal infusion agar for 48 hours, then making a suspension of the growth in sterile physiological salt solution, and diluting this to a reading of 2.4 cm. on the standardizing gauge of Gates (1920).

The results of the experiment are recorded in Table I. A number of the animals died early from an intercurrent pneumonia. The results sought, however, were obtained in a clear-cut manner. All of the animals became infected as evidenced by the recovery of the organism from the spleens. The cultural record for Animal C was misplaced and lost, and decomposition prevented cultural work on Animal B.

Table I shows clearly that white mice may be infected with very minute amounts of cultures of *Bacillus abortus*. The results with the guinea pig indicate that mouse susceptibility to infection with

TABLE I.
White Mice.

Mouse.	Infecting dose.	Autopsied.	Spleen culture.	Remarks.
A	0.5 cc. serum bouillon culture.	Died; 27th day.	Positive, moderate.	Died from pneumonia. <i>Klo-</i> <i>siella</i> infection of kidneys.
B	Suspension 0.5 cc. undiluted.	" 3rd "	None made.	Badly decomposed. Died from pneumonia. No evidence of peritonitis.
C	" 0.5 " 1:1,000 dilution.	" 9th "	Record lost.	Purulent pneumonia.
D	" 0.5 " 1:1,000 "	Killed; 27th "	Positive, luxuriant.	Spleen enlarged $\times$ 3. Other or- gans normal. Agglutinins 1:160.
E	" 0.5 " 1:10,000 "	Died; 26th "	" moderate.	Spleen normal. Slight pneu- monia. <i>Klostrilla</i> infection of kidneys.
F	" 0.5 " 1:10,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged $\times$ 3. Slight pneumonia. Agglutinins 1:320.
G	" 0.5 " 1:100,000 "	Died; 11th "	" good.	Killed by fighting. Slight splenic enlargement. Slight pneu- monia. <i>Klostrilla</i> infection.
H	" 0.5 " 1:100,000 "	Killed; 27th "	" luxuriant.	Spleen enlarged $\times$ 3. Slight pneumonia. Other organs normal.
I	" 0.5 " 1:1,000,000 "	" 27th "	" "	Spleen normal size. Slight pneu- monia. Agglutinins 1:40.
J	" 0.5 " 1:1,000,000 "	Died; 2nd "	" very few colonies.	Wounded by fighting. Very weak when inoculated. No lesions of internal organs.
Guinea Fig 2114	" 0.5 " 1:1,000,000 "	Killed; 27th "	Positive, moderate.	No lesions. Agglutinins 1:40.

this organism compares favorably with that of the latter animal. No experiments have been done with infected tissues, but there is no apparent reason why white mice should not serve successfully as inexpensive substitutes for guinea pigs in diagnostic work on infectious abortion of cattle.

The Susceptibility of Rats and Mice to Bacillus abortus by Ingestion.

The susceptibility of both wild and tame varieties of mice and rats to infection by feeding with *Bacillus abortus* was tested. Cultures and infected tissues were used.

TABLE II.

White Rats.

Rat.	Method of infection.	Material used.	Autop- sied. <i>day</i>	Spleen culture.	Agglu- tinina.	Remarks.
A	Feeding.	Culture.	52nd	—	—	
B	"	Tissue.	52nd	—	—	
C	Inoculation.	Culture.	23rd	+	1:40	No lesions.
D	"	" 1:100 dilution.	26th	+	1:40	" "
E	"	"	52nd	+	1:40	" "
F	Control.	—	52nd	—	—	

The cultures were salt solution suspensions of the growth removed from agar slants which had been incubated for 24 to 48 hours. Approximately 3 cc. of salt solution was used to suspend the growth from a single tube culture. The infected material was the livers of guinea pigs which had been infected with *B. abortus* and killed during the 4th week after inoculation. Although the organs of the body of the guinea pig are richest in the abortion bacilli at this time, the liver is never the seat of large numbers of the organism. The livers used for feeding had all yielded the *B. abortus* in moderate numbers.

The animals were allowed to fast for 24 hours before feeding. The culture suspensions were placed in Petri dishes and small bits of dry bread added to absorb the fluid. When offered to the hungry animals, the soaked bread was always eagerly consumed. The livers were ground to a pasty consistency in a mortar and smeared on pieces of bread. The paste was eagerly licked off the bread by the rats and taken fairly well by the mice. The day after the first feeding food was again withheld, and on the 3rd day the feeding of infected material was repeated as before and with the same materials.

The results of the feeding experiments are given in Tables II to VI inclusive. In each case several animals were inoculated subcutaneously as controls. It will be noted that all inoculated animals became infected, while all of the feeding experiments, with two ex-

TABLE III.

White Rats.

Rat.	Material fed.*	Autopsied.	Spleen culture.	Agglutinins.	Remarks.
		<i>day</i>			
A	Culture suspension, undiluted, 1 cc.	22nd	Cultures contaminated.	1:10	Died from pneumonia. Organs invaded with colon bacilli.
B	Culture suspension, undiluted, 1 cc.	29th	+	—	Very few organisms present in spleen. No lesions.
C	Suspension, 1:10 dilution, 1 cc.	29th	—	—	
D	Suspension, 1:100 dilution, 1 cc.	29th	—	—	
E	Suspension, 1:1,000 dilution, 1 cc.	29th	—	—	

* In this case the bacterial suspensions were pipetted directly into the mouths of the animals, so the exact dosage is known.

TABLE IV.

Gray Rats.

Rat.	Method of infection.	Material used.	Autopsied.	Spleen culture.	Remarks.
			<i>day</i>		
A	Feeding.	Infected tissue.	19th	—	
B	"	Culture.	19th	+	No lesions. Organism recovered from spleen.
C	Inoculation.	" suspension 1 cc.	22nd	+	No lesions. Organism recovered from spleen. Agglutinins 1:1,280.

ceptions, failed. The two exceptions, one a gray rat (*cf.* Table IV, Rat B), and one a white rat (*cf.* Table III, Rat B), received heavy culture feedings. The data on gray rats are limited because of the difficulty of procuring the animals. In Table III, however, it is

demonstrated that the white rat becomes infected by feeding, only if he receives a very heavy dose of the organism; and this is probably also true of the gray rat. The failure, in all cases, of the guinea pig tissue to infect is best explained on this basis.

TABLE V.
White Mice.

Mouse.	Method of infection.	Material used.	Autop-sied.	Spleen culture.	Agglu-tinins.	Remarks.
			<i>day</i>			
A	Feeding.	Infected tissue.	52nd	—	—	
B	"	Culture.	52nd	—	—	
C	"	"	28th	—	—	
D	"	"	28th	—	—	
E	Inoculation.	"	23rd	+	1:20	Enlarged spleen.
F	"	"	52nd	+	—	" "
G	"	" 1:100 dilu-tion.	26th	+	—	" "
H	Control.	—	52nd	—	—	

TABLE VI.
Gray Mice.

Mouse.	Method of infection.	Material used.	Autop-sied.	Spleen culture.	Agglu-tinins.	Remarks.
			<i>day</i>			
A	Feeding.	Culture.	28th	—	—	
B	"	"	28th	—	—	
C	"	"	2nd	—	—	
D	"	"	33rd	—	—	
E	"	"	33rd	—	—	
F	"	"	52nd	—	—	
G	"	Infected tissue.	52nd	—	—	
H	Inoculation.	Culture.	21st	+	1:160	Organs normal.
I	"	"	52nd	+	1:20	" "
J	"	" 1:100 dilu-tion.	52nd	+	—	" "

CONCLUSIONS.

1. White mice are highly susceptible to infection by inoculation with *Bacillus abortus*. The susceptibility appears to be as great as that of the guinea pig, and this animal probably can be substituted satisfactorily for guinea pigs in diagnostic work.

2. Both mice and rats are very refractory to feeding infection with *Bacillus abortus*. The failure to infect mice in this way was complete, but the feeding of large amounts of culture gave infection in rats. Subcutaneous inoculation resulted in infection of all the animals. The difficulty of infecting rats and mice with *Bacillus abortus* by feeding makes it very doubtful whether these animals can have any rôle in the propagation and spread of infectious abortion in cattle.

BIBLIOGRAPHY.

- Ascoli, A., *Z. Infektionskrankh. Haustiere*, 1915, xvii, 156.
Fabyan, M., *J. Med. Research*, 1912, xxvi, 441.
Gates, F. L., *J. Exp. Med.*, 1920, xxxi, 105.
Holth, H., *Z. Infektionskrankh. Haustiere*, 1911, x, 207.
Zwick and Zeller, *Arb. k. Gsndhtsamte*, 1912-13, xliii, 1.

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